No. 86

VOLUME XXII JANUARY 1961

American Journal of

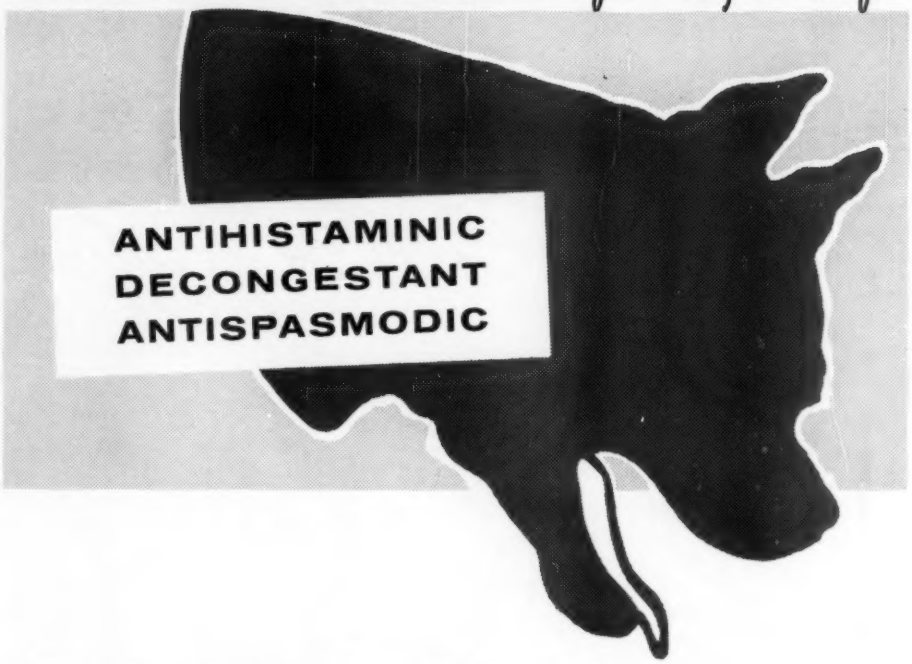
Veterinary RESEARCH

In this issue . . .

- IBR Virus in Cattle with Conjunctivitis 13
- Ovine Vibriosis Immunization 43
- Localization and Effects of Swine Nasal Virus . . 67
- Comparative Study of PPLO and Ornithosis . . . 85

CONTENTS ARE ON PAGES 1, 6, 10

BENYLIN® EXPECTORANT *for cough relief*



Benylin Expectorant is a combination of approved medical agents which assist in liquefying and removing mucous secretions from the respiratory tract.

USEFUL IN ALL COUGHS AND OTHER SYMPTOMS OF CONGESTION ASSOCIATED WITH THE RESPIRATORY SYSTEM

Benylin Expectorant is available in 16-ounce and 1-gallon bottles.

Not recommended for use in animals supplying milk for human consumption.

Complete information available on request.

PARKE-DAVIS

PARKE, DAVIS & COMPANY
DETROIT 32, MICHIGAN

PARKE, DAVIS & COMPANY, LTD.
MONTREAL 9, QUEBEC

208—1160

American Journal of Veterinary Research

Volume XXII | January, 1961 | Number 86

Contents

The articles in the Research Journal are grouped, loosely, by animal species, followed by the more general articles. The articles listed on the cover are those that appear at the beginning of the various divisions or are particularly representative of a number of similar articles in a division.

The Isolation of Infectious Bovine Rhinotracheitis Virus from Cattle Affected with Conjunctivitis—Observations on the Experimental Infection 13
F. R. Abinanti and G. J. Plumer

A Rapid Serologic Test for Rinderpest 18
S. S. Stone and W. M. Moulton

Clinical and Hematologic Investigation of a High Incidence of Lymphosarcoma in a Herd of Cattle 23
G. H. Theilen, O. W. Schalm, and V. Gilmore

The Effect of Diethylstilbestrol and Progesterone on the Growth of Four Mastitis-Producing Bacteria 32
N. A. Frank and W. D. Pounden

Neural Mechanisms in Sexual Behavior. III. Preputial Muscles and Their Reflexes in Bull Calves and Rams 37
L. L. Larson, R. L. Kitchell, and B. Campbell

Experimental Immunization Against Ovine Vibriosis. I. The Use of Live and Formalin-Killed *Vibrio fetus* Vaccines 43
V. A. Miller and R. Jensen

Pingue (*Hymenoxys richardsonii*) Poisoning in Sheep 47
W. A. Aanes

Neuropathology of Ovine and Bovine Toxoplasmosis 53
A. Koestner and C. R. Cole

Site of Localization and Effects of Swine Nasal Virus in Experimental Pigs 67
W. P. Switzer, E. D. Roberts, and C. L'Ecuyer

(Continued on page 6)

PRINTED IN U.S.A.

Council on Research of the AVMA

CARL A. BRANDLY, Urbana, Ill.
GEORGE T. EDDS, Fort Dodge, Iowa
LLOYD C. FERGUSON, East Lansing, Mich.
ROBERT GETTY, Ames, Iowa
WILLIAM A. HAGAN, Ames, Iowa
RUE JENSEN, Fort Collins, Colo.
T. CARL JONES, Boston, Mass.
GEORGE C. POPPENSIEK, Ithaca, N.Y.
C. ROGER SMITH, Columbus, Ohio
RICHARD D. TURK, College Station, Texas

Committee on Journal of the Council on Research

CARL A. BRANDLY, LLOYD C. FERGUSON, WILLIAM A. HAGAN

Reviewers of Research Manuscripts

JOSEPH O. ALBERTS, Urbana, Ill.
JAMES ARCHIBALD, Guelph, Ont.
WILFORD S. BAILEY, Auburn, Ala.
CHARLES N. BARRON, Philadelphia, Pa.
WILLIAM E. BROCK, Stillwater, Okla.
M. LOIS CALHOUN, East Lansing, Mich.
CLARENCE R. COLE, Columbus, Ohio
CHARLES H. CUNNINGHAM, East Lansing, Mich.
DAVID K. DETWEILER, Philadelphia, Pa.
ROBERT W. DOUGHERTY, Ithaca, N.Y.
HOWARD W. DUNNE, University Park, Pa.
JEAN C. FLINT, Fort Collins, Colo.
JOHN R. GORHAM, Pullman, Wash.
DONALD P. GUSTAFSON, Lafayette, Ind.
JAMES A. HENDERSON, Guelph, Canada
WILLIAM KAPLAN, Chamblee, Ga.
ALFRED G. KARLSON, Rochester, Minn.
RALPH L. KITCHELL, St. Paul, Minn.
WENDELL KRULL, Stillwater, Okla.
ROGER P. LINK, Urbana, Ill.
HADLEIGH MARSH, Bozeman, Mont.
ROBERT R. MARSHAK, Philadelphia, Pa.
ERSKINE V. MORSE, Lafayette, Ind.
CARL OLSON, Madison, Wis.
RICHARD L. OTT, Pullman, Wash.
R. ALLEN PACKER, Ames, Iowa
WILLIAM R. PRITCHARD, Gainesville, Fla.
STEPHEN J. ROBERTS, Ithaca, N.Y.
ARNOLD S. ROSENWALD, Davis, Calif.
JOHN H. RUST, Chicago, Ill.
H. R. SEIBOLD, Plum Island, N.Y.
ALVIN F. SELLERS, Ithaca, N.Y.
HERBERT G. STOENNER, Hamilton, Mont.
MELVIN J. SWENSON, Ames, Iowa
WALTER G. VENZKE, Columbus, Ohio
CHARLES K. WHITEHAIR, East Lansing, Mich.

EDITORIAL STAFF: D. A. PRICE, *Editor-in-Chief;* L. MEYER JONES, *Assistant Editor;* EVA G. BAILEY, *Assistant to the Editors.*

Published bimonthly at 600 S. Michigan Ave., Chicago, Ill., by the American Veterinary Medical Association. Second class postage paid at Chicago, Ill., and at additional mailing offices. Reproduction of any part of this publication is prohibited unless special permission is given by the publishers and the authors. Subscription price: $15.00 per year in the United States; Canada, $15.50; Pan American countries, $16.50; foreign countries, $17.00. Members of the American Veterinary Medical Association may subscribe to the American Journal of Veterinary Research for $5.00 per year added to the annual dues of $25.00. Single copies, $2.50. Address all correspondence to American Veterinary Medical Association.

Change of address: Send new address (and moving date) 45 days before moving. Include old address as it appeared on previous issue (if possible send label). Do not depend on the post office to forward the JOURNAL.

DIFCO

Antisera and Antigens

Enteropathogenic Coli Klebsiella

Salmonella Shigella

Brucella Leptospira Streptococci

*

C Protein Antiserum and Standard

*

Infectious Mononucleosis Antigens and Standards

Potent

Stable

Specific

Descriptive literature available on request

Specify

DIFCO LABORATORY PRODUCTS

BIOLOGICS CULTURE MEDIA REAGENTS

new
250 ml. Sterile Solution
Veterinary
ERYTHROCIN®
injectable
Erythromycin Ethyl
Succinate for Injection,
Containing Erythromycin
12.5 Gm.
ABBOTT
20cc
15
10
5

NEW

Erythrocin®

INJECTABLE VETERINARY

for large animals...for small animals...for poultry

Erythrocin Injectable Veterinary is a safe, fast-acting antibiotic with a wide range of indications in both large and small animals.

Fast Blood Levels

Erythrocin Injectable administered at therapeutic dosage will provide peak blood levels in one to two hours, with effective concentration remaining in the system for several hours.

Effective against "Resistant" Strains

Studies have shown Erythrocin Injectable effective against staph, strep, and pneumococci—*including some strains which have become resistant to the action of other antibiotics.*

Safe

Erythrocin has an unparalleled record of safety in use in human medicine. Clinical trials on over 1900 large and small animals indicate that Erythrocin Injectable Veterinary also has a high order of safety. In addition, there are seldom complications with Erythrocin therapy and little likelihood of resistance developing during, or as a result of, treatment.

®Erythrocin—Erythromycin, Abbott

Bactericidal against some Common Invaders

Erythrocin is classified as bactericidal, against certain coccal organisms. This means prompt clinical results for your patients.

Many indications in Veterinary Practice

Some typical large and small animal conditions for which Erythrocin may be successfully used, include: pneumonia, upper respiratory infections, "shipping fever", mastitis, metritis, "foot rot", wound infections, pleurisy, "lamb dysentery", secondary infections (as in canine distemper), and as an aid in the management of leptospirosis in sows at farrowing and of some "scours" in young pigs.

Available in two Sizes

Erythrocin Injectable Veterinary (List No. 8589) comes in 250 and 100 ml. bottles. It is stable at normal room temperature and retains its potency indefinitely.

ABBOTT

011250

(Contents—Continued from page 1)

Post-Tetanic Potentiation in Spinal Cord of Normal and Myoclonic Pigs 72
M. W. Stromberg, R. L. Kitchell, and R. C. Callstrom

Correlation of the Effects of Parathion on Erythrocyte Cholinesterase with Symptomatology in Pigs 80
W. T. Oliver and H. S. Funnell

The Histopathologic Characteristics of Diseases Producing Inflammation of the Air Sacs in Turkeys—A Comparative Study of Pleuropneumonia-like Organisms and Ornithosis in Pure and Mixed Infections 85
J. N. Beasley, R. W. Moore, and J. R. Watkins

Newcastle Disease Immunity in Broilers and Caponettes Vaccinated with Killed-Virus and Live-Virus Vaccines 93
A. H. Dardiri, V. J. Yates, P. W. Chang, and D. E. Fry

Enzyme Activity Levels in Chicken Plasma 99
L. S. McDaniel and H. L. Chute

Epizootic Hemorrhagic Disease in White-Tailed Deer—Characteristics of the South Dakota Strain of Virus 104
E. C. Pirtle and J. M. Layton

Studies in Anaplasmosis. II. Electron Microscopy of *Anaplasma marginale* in Deer 109
M. Ristic and A. M. Watrach

The Etiology of Legume Bloat—Nonvolatile Acids 117
W. G. Rosen, H. Fassel, and R. E. Nichols

Studies on Infections and Immunity with the Lungworm, *Dictyocaulus viviparus* (Bloch). I. Active Immunization of Guinea Pigs and Rabbits 123
A. E. Wade, L. E. Swanson, and L. E. Fox

Studies on *Trichostrongylus axei* (Cobbold, 1879). VII. Some Quantitative and Pathologic Aspects of Natural and Experimental Infections in the Horse 128
S. E. Leland, J. H. Drudge, Z. N. Wyant, and G. W. Elam

Recovery of Western Equine Encephalomyelitis Virus from Crop Washings of Experimentally Infected Pigeons 139
J. F. Winn and D. F. Palmer

(Continued on page 10)

High-Speed . . . . . DEPENDABLE PRESSURE STEAM STERILIZATION

AMERICAN STERILIZER
613-R PORTABLE DYNACLAVE

• The low cost, high performing American 613-R Dynaclave assures positive sterilization with pressure steam at 250° F. or 270° F. It is fast, reaching 270° F. in approximately seven minutes.

Operation is fully automatic with selective sterilizing cycles from 3 to 60 minutes. Cools and dries instruments or supplies by exhausting steam and residual water back into water reservoir—NOT into room.

The 613-R, with greater capacity, accommodates three large trays (6" x 13"). Handsome, all stainless steel construction is durable and easy to clean. Other features include a Safety-Lock Door, Adjustable Thermostat and Accurate Temperature Gauge. Automatically burn-out proof.

See your authorized American Sterilizer dealer or write for Bulletin DC-410.

AMERICAN STERILIZER
Erie • Pennsylvania

IN CANADA: The American Sterilizer Company of Canada Limited, Brampton, Ontario

Date of Receipt to Be Published

Beginning with the January, 1961, issue of the *A.J.V.R.*, the date received (date on which manuscript first arrives at the editorial office) will be indicated by a footnote on each manuscript published. In order to receive this dating, manuscripts must be prepared in accordance with the instructions to authors.

After the date received is assigned to a manuscript, it is sent to a reviewer representing the AVMA Council on Research. If the reviewer does not recommend acceptance, the manuscript is returned to the author promptly, usually within three weeks of the date received. If the reviewer recommends publication, the manuscript will be edited in turn, then sent back to the author for revision with a copy of the reviewer's comments. If the revision is acceptable, the author is notified and the manuscript prepared for printing. The author next receives the galley proof and a reprint order form.

On occasion, the reviewer or the editor may feel that the opinion of a second reviewer is needed. In such cases, the author is given a copy of the comments of each reviewer. It is the editor's responsibility to adjudicate differences of opinion and to rule on final acceptance.

Instructions to Authors

American Journal of Veterinary Research

All manuscripts submitted for publication in the *American Journal of Veterinary Research* are sent to reviewers especially qualified in the various fields. If accepted, it is understood that the AVMA has exclusive publication rights.

Manuscripts, including footnotes, references, and tables should be typewritten, double-spaced, on 8½- by 11-in. bond paper, with ample margins. The original and one carbon copy should be submitted (the carbon should be clearly legible so it can be used for marking by the reviewer, if he chooses to do so). Articles should be concise and should avoid technical jargon and complex sentences.

Photographs should be furnished in glossy prints of a size that will fit into the pages of the Research Journal with a minimum of reduction. Photomicrographs which cannot be reduced should be marked for cropping to 1- or 2-column width. Identifying marks such as arrows, letters, or numbers, should be clearly marked to insure good contrast for reproduction and must be large enough to stand reduction, if necessary.

Drawings, graphs, and charts should be made clearly and accurately with India ink on white paper and a glossy print submitted when possible. Numbers or letters should be large enough to allow for any necessary reduction of the graph or chart. Blue lines in graph paper drop out in reproduction; therefore, if lines are required they must be drawn in black ink. All illustrations should bear the name of the author and the illustration number written *lightly* on the back with lead pencil.

Tables should be simple and typed double space. Complex tables discourage perusal. It is better to summarize complex material than to tabulate it.

References should be typed double space, in alphabetical order by author, in a style used in recent issues of the Research Journal. All references, particularly personal communications (name, location, date) and unpublished data (authors, title, journal), must be complete so that anyone wishing to do so may verify them.

The author will receive a galley proof of his article, which he should check carefully. With his galley proof, he will be sent a rate sheet and an order blank for reprints, which must be ordered within 30 days after publication.

New Lower Price plus New 25-Capsule Bottle

BYOGIC®

(ANTIBACTERIAL—ANTICHOLINERGIC)

for the Treatment and Management of the Respiratory-Enteritis Complex and Sequelae

Byogic is a combination of two specific agents, Erythrocin® and Tral® for the treatment of upper respiratory infections and enteric disturbances in small pet animals and laboratory colonies.

Ingredients of Proven Safety and Effectiveness

Each Byogic capsule contains 100 mg. Erythrocin, an antibiotic of demonstrated safety and one that is quite often effective against "resistant" organisms.

Each capsule also contains 20 mg. of Tral, our highly specific anticholinergic that acts directly on the gastro-intestinal tract to reduce secretion of digestive juices and the motility of the stomach and intestines.

Especially Valuable in Treatment of Puppies and Old or Debilitated Animals

Gastro-intestinal involvement often accompanies infectious conditions in young, old, or weak animals. The antibacterial-anticholinergic action of Byogic makes it possible to treat complicated conditions in these animals quickly, safely, and inexpensively. **Complete literature and clinical data is yours for the asking, doctor.**

ABBOTT

BYOGIC—ERYTHROCIN WITH TRAL ERYTHROCIN—ERYTHROMYCIN STEARATE, ABBOTT TRAL—MEDOCYCLIUM METHYLSULFATE, ABBOTT 101195

(Continued from page 6)

The Position of the Canine Prostate Gland 142
N. Gordon

Use of Emulsified Fat for Intraperitoneal Alimentation 147
M. J. Burns and C. H. Clark

Oxyuriasis in Laboratory Mouse Colonies 150
W. G. Hoag

Scientific Notes 154

Study of Chicken Sarcoma (Strain 13) Placed in the Bursa of Fabricius—*E. L. Stubbs and F. G. Sperling* 154

Cardiac Output and Related Values in Anesthetized Deer—*A. L. Good, C. M. Stowe, and L. Blankenship* 156

Exsanguination of Chickens by Catheterization—*A. A. Hirata* 158

Research Tools 159

Classified Advertisements

The American Journal of Veterinary Research accepts classified advertisements from graduate veterinarians seeking veterinary technological positions with colleges, universities, and other institutions such as industrial and commercial research laboratories.

Also, classified advertisements are accepted from institutions and other organizations which may be seeking veterinary bacteriologists, parasitologists, pathologists, or other veterinary technological personnel.

This service is intended to facilitate the placement or finding of personnel with specialized training in veterinary medical science.

The rate for these classified advertisements is $4.50 for the first 25 words and 15 cents for each additional word. No charge is made for use of a box number.

The closing date for classified advertisements in this Journal (a bi-monthly) is the 20th of the month two months preceding date of issue.

Bind Your Copies

of the

JOURNAL OF THE AMERICAN VETERINARY MEDICAL ASSOCIATION

and

AMERICAN JOURNAL OF VETERINARY RESEARCH

The publishers have effected an arrangement with one of America's foremost bookbinders by which subscribers can have their journals economically bound, to the publisher's specifications.

Your copies of the AMERICAN JOURNAL OF VETERINARY RESEARCH will be bound in the finest grade blue washable buckram, with your name imprinted on the front cover. Price $4.45.

Ship journals parcel post. Bound volumes will be returned transportation prepaid. Full remittance must accompany order.

The Book Shop Bindery

Publisher's Authorized Binding Service

Creators of Distinctive Bindings
Since 1895

5809 W. Division St., Chicago 51, Illinois

FOR CALF SCOURS

ENTEFUR®

BOLUS VETERINARY, SMALL

COMBINES

FURAMAZONE (1 Gm.)—for specific antibacterial activity against gram-negative and gram-positive enteric bacteria

BISMUTH SUBSALICYLATE (0.26 Gm.)—for astringent, antidiarrheal activity, and as a protective to the lining of the gastrointestinal tract

TO PROVIDE

"UNIFORMLY EXCELLENT RESULTS..."

Bull, W. S.: N. Am. Vet. 38:3 (Jan.) 1957.

Dosage: 1 bolus perorally, twice daily, for 2 or 3 days.
Supplied: Boluses of 3.3 Gm. each, in boxes of 24 and 500.

EATON LABORATORIES
Division of The Norwich Pharmacal Company
NORWICH, NEW YORK

American Journal of Veterinary Research

Established 1940

COPYRIGHT 1961 BY AMERICAN VETERINARY MEDICAL ASSOCIATION

VOL. XXII JANUARY, 1961 No. 86

The Isolation of Infectious Bovine Rhinotracheitis Virus from Cattle Affected with Conjunctivitis—Observations on the Experimental Infection

F. R. ABINANTI, D.V.M., Ph.D., and G. J. PLUMER, D.V.M.

Bethesda and College Park, Maryland

INFECTIOUS bovine rhinotracheitis virus (IBR) has been established as the causative agent of a respiratory disease of cattle, characterized by severe inflammation of the upper respiratory tract; copious, serous nasal discharge; salivation; dyspnea; and fever.[2] This same virus also has been shown to cause infectious pustular vulvovaginitis in cattle.[1]

In a more recent report on IBR infections of cattle, McKercher *et al.*[3] stated that conjunctivitis is seen with and without accompanying respiratory signs. They speculated on the possibility that conjunctivitis may represent the major clinical manifestation of the infection. The present report deals with an epizootic of conjunctivitis in feeder cattle that had no accompanying respiratory signs, although IBR virus was recovered. Studies are also reported on conjunctival exposure of calves to IBR virus and to the virus isolated from the cattle with conjunctivitis.

MATERIALS AND METHODS

The affected cattle from which the virus was recovered will be described along with the clinical data. Specimens of ocular and nasal secretions were taken from 4 of the affected cattle. The secretions were collected with cotton-tipped applicator sticks which were then whirled in vials containing 4 ml. of Hanks' balanced salt solution (BSS) containing 0.5 per cent gelatin and antibiotics (each milliliter contained 100 units of penicillin, 100 μg. of streptomycin, 100 units of tetracycline, and 100 units of nystatin). Two milliliters of the specimen were inoculated into tissue cultures of primary bovine embryonic kidney cells* for virus isolation attempts. Blood specimens also were taken from these same 4 cattle during the acute and convalescent stages.

Calves were inoculated with virus recovered from 1 of the cattle with conjunctivitis (10051) and with a California isolate of IBR virus to compare the types of infection produced by each and to procure serum for cross-neutralization tests. Four 3- to 6-week-old calves were used for these experiments. The control animal was a yearling Aberdeen Angus bull. Two calves were inoculated with each of the viruses and kept in pairs in double-doored stalls at opposite ends of an isolation barn. The control animal was housed in a separate isolation stall. Separate personnel cared for each of the groups of animals, and footwear was cleaned and disinfected when personnel entered and left the isolation stalls.

The calves were infected by dipping a cotton-tipped applicator into the virus suspension and twirling it against the cornea while holding the lid firmly closed over the eye. The tissue culture infectivity titer of the 10051 virus inoculum was $1 \times 10^{5.5}$, while that of the IBR virus was $1 \times 10^{7.0}$. The opposite eye was similarly treated with normal tissue culture fluid (Hanks' BSS), as were both eyes of the control animal. Ocular and nasal secretions, fecal swabs, and pre- and postinoculation serums were collected from all 5 experimental cattle and processed in a manner similar to that described for the naturally infected cattle. The ocular and nasal secretions and fecal swabs were taken prior to inoculation and at two- to three-day intervals for 14 days thereafter. Rectal temperatures were

Received for publication Jan. 18, 1960.

From the U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Infectious Diseases, Bethesda, Md. (Abinanti), and the Maryland State Board of Agriculture, Live Stock Sanitary Service Laboratory, University of Maryland, College Park (Plumer).

* Procured from Microbiological Associates, Bethesda, Md.

recorded on the day of inoculation and daily thereafter for the next 17 days. Specimens of blood were collected at the time of inoculation and 7, 21, and 39 days following inoculation.

The IBR virus used for the neutralization tests and for inoculating the 2 calves was a California isolate* which had been passaged in tissue culture three times in this laboratory. The low infectivity titer of the virus pool used in these studies conceivably was the result of improper storage.

The virus isolated from 1 of the cattle with conjunctivitis has been designated 10051. This virus was used for the neutralization tests and for infecting the calves and represents the third tissue culture passage of the original isolate.

TABLE 1—Antibody Response of Cattle with Conjunctivitis when Tested by the Neutralization Technique Against Virus Recovered from Their Ocular Secretions

Cattle No.	Serums*	
	Acute phase	Convalescent phase
336	None	100**
19340	None	None
481	None	>100
18826	10	>100
Kerato-conjunctivitis	None	None
Uninfected	None	.....

* Serums were diluted 1:2. ** Number of tissue culture doses ($t.c.i.d._{50}$) of virus neutralized by serum.

The neutralization tests were performed by the virus dilution technique that is usually utilized for obtaining a virus neutralization index.[4] Neutralization could not be demonstrated when the usual 100 $t.c.i.d._{50}$ was used with dilutions of serum. In these experiments, dilutions of the virus were mixed with a single fixed dilution of serum (1:2 in some tests and 1:4 in others). The serum-virus mixtures were incubated at room temperature for one hour and then inoculated into two tubes of bovine embryonic kidney tissue cultures. The serum protection titer was expressed as the difference in virus titer observed between serums obtained from cattle in acute and convalescent stages, or the difference seen between normal versus specific immune serums.

The identity of the virus recovered from the cattle with conjunctivitis was established by means of neutralization tests, using IBR hyperimmune rabbit serum.

RESULTS

CLINICAL AND EPIDEMIOLOGIC OBSERVATIONS

In November, 1958, severe conjunctivitis without any other sign was observed in a herd of 125 yearling Aberdeen Angus steers that had been assembled over a period of two weeks from three auction markets. At the time of purchase, there was some lacrimation; however, it was not until six weeks after the animals arrived at the feedlot that any significant number of cattle began to lacrimate profusely. A week later, two thirds of the cattle were affected.

The palpebral conjunctiva was intensely swollen and covered with fine petechial hemorrhages. The swelling was great enough in many of the cattle to cause the conjunctiva to protrude from under the lid. The bulber conjunctival vessels were injected. The hair over most of the cheek was soaked with the ocular secretion. The discharge varied from serous to purulent, but was predominantly serous. Both eyes were involved in many individuals; in some, one eye was lacrimating profusely, while the other had a thick, creamy exudate. Ulcers or corneal opacities were not seen. At the corneal limbus of some, a faint but distinct white ring was visible. Most of the lacrimating cattle also had a slight to moderate clear nasal discharge. The body temperatures were normal in animals in which it was measured. No respiratory signs were observed by the clinician (G.J.P.) during the height of the epizootic nor were any observed by the herdsman prior to or following this period.

Copious lacrimation and visible swelling of the lids persisted in individuals for about a week. Signs were not apparent in any animals in the herd three weeks after the first cases appeared.

Casual clinical observation of this occurrence of conjunctivitis might have classified it as bovine infectious keratitis (BIK). However, the disease differed from BIK in several obvious characteristics. It was primarily an infection of the ocular and palpebral conjunctiva. The cornea was not consistently involved and, when it was, the changes were mild, did not include ulcers, and were confined to the limbus. The discharge was primarily serous in character.

A cytopathogenic agent was recovered, using tissue cultures of bovine embryonic kidney, from the ocular and nasal secretions of all 4 of the cattle tested. Serum samples taken from these same 4 cattle during the acute and convalescent stages were tested by the tissue culture neutralization technique against one of the agents recovered from the ocular secretions (10051). The results are presented (table 1). In 3 of the 4 cattle, serum taken at the convales-

* Obtained from Dr. C. J. York, Pitman-Moore Co., Indianapolis, Ind.

cent stage neutralized 100 t.c.i.d.$_{50}$, or more of the virus than did serum taken during the acute stage. Serum from a dairy cow which was convalescing from bovine infectious keratitis gave no protection against this virus, nor did serum from a normal cow.

Fortunately, at this time, work with IBR virus was being done in our laboratory, and a similarity in the cytopathic effect of the two viruses was observed. Accordingly, IBR hyperimmune rabbit serum, serum from cattle in acute and convalescent stages of IBR, and serum from a steer at the acute and convalescent stages of conjunctivitis were tested by neutralization against IBR virus and an isolate (10051) from 1 of the affected cattle. The results are presented (table 2). Each of the serum samples taken during the convalescent stage and the postinoculation IBR rabbit serum gave reciprocal protection against each of the viruses. Therefore, it would appear that the virus recovered from the cattle with conjunctivitis is IBR virus.

TABLE 2—Serologic Results Demonstrating the Relationship of IBR Virus to the Virus Recovered from Cattle Affected with Conjunctivitis

Time of serum sampling	Neutralization against 10051	Neutralization against IBR
IBR RABBIT SERUM		
Preinoculation	None	None
After hyperimmunization	13*	>13
IBR CATTLE SERUM		
Acute phase	None	None
Convalescent phase	>100	>10
CONJUNCTIVITIS		
Acute phase	None**	None
Convalescent phase	13	>13

* Number of tissue culture doses (t.c.i.d.$_{50}$) of virus neutralized by serum. ** From present study.

OBSERVATIONS ON EXPERIMENTALLY INFECTED CALVES

Two 3- to 6-week-old dairy calves were inoculated interpalpebrally with the virus recovered from 1 of the cattle with conjunctivitis (10051) and 2 were given IBR virus to compare the type of infection produced by each and to obtain serums for cross-neutralization studies. The clinical observations and virus isolations for this group of calves are summarized (table 3).

Calf 806, exposed to the isolate 10051, on postinoculation day 3 had a profuse serous discharge from the eye inoculated with the virus; by day 4, there was considerable edema of the palpebral conjunctiva and its surface was stippled with fine hemorrhages. Lacrimation reached its peak by day 5 and continued through day 14. A thick, creamy exudate was visible on day 7. The uninoculated eye appeared normal during the 21 days the calves were observed. Virus was recovered from the ocular and nasal secretions for seven days after inoculation, but only from the virus-infected eye.

The response of the second calf (807), exposed to the same virus, differed from that of calf 806 in that the thick exudate appeared on day 2 and persisted through day 12. Lacrimation was less severe, but edema and congestion of the conjunctival blood vessels were more pronounced. The excretion of virus was similar to that of

TABLE 3—Summary of Clinical and Virologic Observations of Calves Experimentally Infected by Interpalpebral Inoculation with 10051 and IBR Viruses

Calf No.	Clinical and virologic findings	Days following inoculation: 2	3	4	5	6	7	9	12	14
806 (10051 virus)	L	....	+++	+++	++++	++++	++	++	++	++
	E	....	....	....	....	....	+	....	....	....
	E & C	....	....	+	++	+++	+	+	....	....
	V	+	NT	NT	+	NT	+	....	....	....
807 (10051 virus)	L	....	+	+	++	++	+	+	+	....
	E	+	+	+	+	+	+	+	+	....
	E & C	....	+	+	+++	+++	++	+	+	....
	V	+	NT	NT	+	NT	+	+	....	....
810 (IBR virus)	L	....	....	+	+	+	+++	++	+	....
	E	....	....	....	....	....	++	+	....	....
	E & C	....	....	....	....	....	++	+	....	....
	V	....	NT	NT	+	NT	+	....	....	....
809 (IBR virus)	No clinical signs*	....	....	....	....	....	....	*+	....	+
808 (normal control)	No clinical signs	....	....	....	....	....	....	....	....	....

\+ to ++++ = degree of severity; L = lacrimation; E = exudate; E & C = edema and congestion; V = virus recovered (+); NT = not taken.
* Virus was recovered from the uninoculated eye on days 9 and 14.

calf 806, but persisted through day 9. The uninoculated eye remained normal.

Only 1 of the calves (810) given the IBR virus developed signs of infection. Lacrimation began on day 4 and continued through day 12. There was not the degree of ocular irritation seen in the calves given the 10051 strain. Virus was recovered from the inoculated eye and from the nasal secretions from days 4 through 7.

Calf 809 did not respond clinically and, it would appear, did not become infected from the inoculation. However, virus was recovered from the uninoculated eye and nasal secretions during postinoculation days 9 to 14, indicating contact infection from its penmate rather than infection resulting from the experimental inoculation.

The control bull (808), given Hanks' BSS intrapalpebrally, developed no clinical evidence of conjunctivitis, nor was virus recovered from the ocular or nasal secretions. This bull had no pre-existing neutralizing antibody for IBR, and none was demonstrable 19 days following inoculation of the calves.

Several of the cattle had body temperatures above 103.5 F. Calf 807, inoculated with 10051 isolate, had a temperature of 104.0 F. on postinoculation days 5 and 6. Calf 810, the only IBR-inoculated steer that developed a conjunctivitis, had temperatures of 103.6, 104.1, 103.6, and 103.8 F. on days 12 through 15, respectively. At this time, the animal had ceased to excrete detectable virus. The control bull had a body temperature of 103.7 F. on day 13, but as observed earlier, did not develop any detectable evidence of infection with either virus.

TABLE 4—Neutralization Results on Serum Samples from Calves Experimentally Inoculated with the 10051 Isolate and the California Isolate of IBR

Calf No.	Virus	
	10051	IBR
806	None*	None
(10051 isolate)	100**	100 or greater
807	None	None
(10051 isolate)	100 or greater	100 or greater
810	None	None
(IBR virus)	100	10–100
809	None	None
(IBR virus)	None	None
808	No neutralization with any of the serum samples from this animal	
(control)		

* Preinoculation. ** Postinoculation number of t.c.i.d.$_{50}$ neutralized.

Nasal discharge in all the animals was so slight and variable that it was difficult to record as a sign. Corneal ulcers or opacities were not observed.

Cytopathogenic agents were not recovered from any of the fecal swabs taken from the 5 animals.

SEROLOGIC RESPONSE

Serum samples were collected from the calves on the day of inoculation and 7, 21, and 39 days following inoculation. Blood was obtained from the control bull (808) on days 7 and 21. These serum samples were tested by neutralization with the 10051 isolate and the California isolate of IBR virus; the results are compiled (table 4). None of the animals had evidence of pre-existing specific neutralizing antibody. The 2 calves given the 10051 isolate of IBR virus (806 and 807) developed antibody to the California isolate earlier than they did to the homologous strain.

Calf 810, inoculated with the California isolate of IBR virus, developed antibodies which afforded equal protection against both viruses. As mentioned earlier, even though virus was recovered from calf 809, no demonstrable specific neutralizing antibody was observed in serum samples collected as long as 29 days after onset of virus excretion.

The control bull had no pre-existing specific neutralizing antibody, and none was demonstrable after 21 days.

These serologic results furnish additional evidence that the IBR virus and the virus recovered from the cattle with clinical cases of conjunctivitis are the same or closely related viruses.

DISCUSSION

This report of conjunctivitis in cattle infected with IBR virus substantiates the observations and speculations made by McKercher *et al.*[3] relative to the occurrence of conjunctivitis as the more prevalent or only sign resulting from infection with the virus. The random distribution in feedlots of cases of respiratory disease attributed to IBR infection may represent only the more obviously sick cattle; the majority of animals may have only conjunctivitis or, as in calf 809, have a subclinical infection.

Infectious bovine rhinotracheitis may produce respiratory disease, pustular vulvovaginitis, or, as described here, conjuncti-

vitis without respiratory involvement. Such diverse pathogenicity, however, is not uncommon. Adenovirus 3 infection in man, for example, can cause conjunctivitis, pharyngoconjunctival fever, acute respiratory disease, pneumonia, or simple pharyngitis.[4] The reasons for such protean effects are not known. In the light of our present knowledge, however, it would seem that thought should be given to revising the restrictively descriptive name of infectious bovine rhinotracheitis, a name which will cause an unnecessary amount of confusion among those people working in the field of infectious diseases.

Summary

Conjunctivitis was observed in a group of 125 yearling beef cattle, approximately two thirds of which were affected. Clinically, it simulated keratoconjunctivitis or "pink eye," but there were no corneal opacities or ulcers. Respiratory signs were not observed. Four isolates of infectious bovine rhinotracheitis (IBR) virus were recovered from the ocular and nasal secretions. Experimental conjunctival inoculation of calves with one of these isolates and with a California isolate of IBR virus resulted in production of conjunctivitis; postinoculation serum samples had reciprocal neutralizing antibodies against each virus.

References

[1] Kendrick, J. W., Gillespie, J. H., and McEntee, K.: Infectious Pustular Vulvovaginitis of Cattle. Cornell Vet., 48, (1958): 458–495.

[2] Madin, S. H., York, C. J., and McKercher, D. G.: Isolation of the Infectious Bovine Rhinotracheitis Virus. Science, 124, (1956): 721–722.

[3] McKercher, D. G., Saito, J. K., Wada, E. M., and Straub, O.: Current Status of the Newer Virus Diseases of Cattle. Proc. U.S. Livestock San. Assoc., (1958): 136–158.

[4] Rowe, W. P., Huebner, R. J., Hartley, J. W., Ward, T. G., and Parrott, R. H.: Studies of the Adenoidal-Pharyngeal-Conjunctival (APC) Group of Viruses. Am. J. Hyg., 61, (1955): 197–218.

Summario in Interlingua

Le Isolation de Virus de Infectiose Rhinotracheitis Bovin ab Bestial Afficite de Conjunctivitis.

Observationes Relative al Infection Experimental

Conjunctivitis esseva observate in un gruppo de 125 pecias de bestial de carne de un anno de etate. Approximativemente duo tertios del animales esseva afficite. Clinicamente le condition simulava ceratoconjunctivitis o ophthalmia periodic, sed nulle opacitate del cornea esseva notate e nulle ulceres. Nulle signos respiratori esseva observate. Quatro isolatos de virus de infectiose rhinotracheitis bovin esseva obtenite ab le secretiones ocular e nasal. Le inoculation conjunctival experimental de vitellos con un de iste isolatos e con un isolato californian de virus de infectiose rhinotracheitis bovin resultava in le production de conjunctivitis. Specimens de sero obtenite post le inoculation contineva anticorpore neutralisatori reciproc contra le viruses.

A Rapid Serologic Test for Rinderpest

S. S. STONE, Ph.D., and W. M. MOULTON, V.M.D.

Greenport, Long Island, New York

THE DIAGNOSIS of rinderpest has depended upon inoculation of tissue from suspected animals into susceptible and immune cattle and subsequent observation for signs of the disease. However, due to the high morbidity and mortality associated with rinderpest in susceptible cattle population, a rapid diagnostic test is needed.

The application of a complement-fixation (CF) test has been hampered by the extreme anticomplementary activity of tissue extracts used as antigen for the test. However, in a recent publication, Nakamura [9] described a CF test for rinderpest using a sodium hydroxide extract of dried lymph nodes, while Boulanger [2] reported the use of an aqueous extract of acetone-ether-treated spleen as antigen. Both of these methods require prolonged preparation of tissue before it can be used as antigen.

This report describes a procedure for the rapid preparation of tissue antigen and its application in a CF test, using antigen and complement dilution methods.

MATERIALS AND METHODS

Virus.—The three bovine rinderpest viruses are immunologically similar and are named after the geographic site of isolation. Grade Hereford cattle used in this work were infected with Kabete O, Pendik, and Pak Chong (Buffalo) strains of rinderpest virus. A lapinized strain (Nakamura III) was used to produce hyperimmune rabbit serum.

Antiserum.—New Zealand white rabbits were infected by intravenous inoculation of 1 ml. of Nakamura III lapinized rinderpest virus suspension, containing approximately 100 infective doses.[7] Three additional weekly inoculations were given, and blood samples were obtained from the rabbits 8, 12, and 14 days after the last inoculation. Serums were pooled and stored at −40 C. Before use, the pooled serum was diluted and heated at 56 C. for 30 minutes. Control serum was collected from normal rabbits.

Diluents.—Extracts were obtained by homogenizing tissue with an equal weight of phosphate buffer composed of 5.52 Gm. of $NaH_2PO_4 \cdot H_2O$ and 9.56 Gm. of Na_2HPO_4 per liter, giving a pH of 6.8 and an ionic strength of 0.15. This pH and tonicity has been shown to be optimal for virus survival.[8] In the CF tests, a veronal-buffered saline (VBS), pH 7.2, containing 0.1 per cent gelatin, was used.[11]

Preparation of Antigen.—Mesenteric lymph nodes were excised from rinderpest-infected steers during pyrexia, usually four to eight days postinoculation. The nodes were trimmed of excess fat, minced with scissors, and homogenized at 0 C. in a high-speed blender with an equal weight of phosphate buffer, pH 6.8, for 10 minutes. This slurry was centrifuged at 0 to 5 C. for 30 minutes at approximately 3,000 x g. After centrifugation, three distinct layers were visible in the tube: a fatty cake at the top, a semi-opaque brown liquid in the center, and a heavy precipitate at the bottom. The center portion was carefully removed to avoid contamination with the top or bottom layers and centrifuged at 10,000 x g for 30 minutes. A similar layering resulted with lesser amounts of the first and third components. The center portion, a 1:2 dilution of the original tissue, was carefully removed and further diluted for testing. Agar-gel diffusion studies, using this antigen in dilutions up to 1:32, produced specific precipitin lines with hyperimmune rabbit serum.[10] Mesenteric lymph nodes collected from normal cattle were treated as described and used as controls.

Complement.—Pooled, fresh, guinea pig serum stored at −40 C. was used as complement (C′).

Indicator System.—Sheep erythrocytes (r.b.c.) were prepared as a 5 per cent suspension, standardized spectrophotometrically as described by Marucci,[7] and sensitized with an equal volume of the appropriate dilution of rabbit hemolysin according to the method of Osler.[11]

Calibration of Complement-Fixation Tubes.—Rimless culture tubes, 7.5 by 100.0 mm., were calibrated at 541 mμ in a Beckman DU spectrophotometer, using hemolyzed r.b.c. All tubes that varied more than 5 per cent in optical density were discarded.

Agar Diffusion.—The agar double-diffusion precipitin technique, as described by White [10] and modified by substituting phosphate buffer as diluent, confirmed the presence of specific antigen. The end point was the highest dilution in which

Received for publication Feb. 5, 1960.

From the Plum Island Animal Disease Laboratory, Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, Greenport, Long Island, N.Y. Dr. Moulton is assigned to the Laboratory from the Animal Disease Eradication Division.

The authors thank Dr. T. L. Barber, for supplying test material, and P. Mikicuik and M. Appelt for technical assistance.

the test sample formed a continuous precipitin line with the positive control.

Complement Fixation by Antigen Dilution.—The procedure used was essentially that of Marucci,[7] employing 0.4-ml. volumes of both serum and antigen and 0.5 ml. of C′ containing five 50 per cent hemolytic units. Titration in the presence of antigen was carried out with one hour of fixation at 37 C., as well as overnight at 5 C. After the fixation period, 0.2 ml. of sensitized r.b.c. was added and the suspension was incubated at 37 C. for 30 minutes. All tubes were centrifuged at approximately 1,000 x g for 10 minutes, and the degree of hemolysis was estimated by visual comparison to tubes containing 25, 50, and 75 per cent of the completely hemolyzed cells used in the test. Tubes with 50 per cent hemolysis were designated 2, the end point; 0, 1, 3, and 4 correspond to 0, 25, 75, and 100 per cent hemolysis, respectively.

A block titration was prepared with tissue antigen and hyperimmune rabbit serum (table 1).

TABLE 1—Block Complement Titration of Rinderpest Antigen and Rabbit Rinderpest Hyperimmune Serum with Fixation Period of 18 Hours at 5 C.

Serum dilution	Antigen dilution				
	1:40	1:60	1:80	1:100	1:120
1:20	0	0	0	0	0
1:40	0	0	0	0	2
1:60	0	1	1	2	3
1:80	0	2	3	4	4
1:100	1	2	3	4	4
1:120	1	2	3	4	4
1:140	2	3	4	4	4
1:160	2	4	4	4	4
1:180	3	4	4	4	4
1:200	3	4	4	4	4
1:220	4	4	4	4	4

ANTICOMPLEMENTARY TITER OF SERUM AND ANTIGEN

Hemolytic units	$1C'H_{50}$	$2C'H_{50}$	$3C'H_{50}$	$4C'H_{50}$	$5C'H_{50}$
Serum dilution 1:40	1	3	4	4	4
Antigen dilution 1:40	1	4	4	4	4

Lysis recorded as: 0 = no lysis, 1 = 25 per cent, 2 = 50 per cent, 3 = 75 per cent, 4 = 100 per cent.

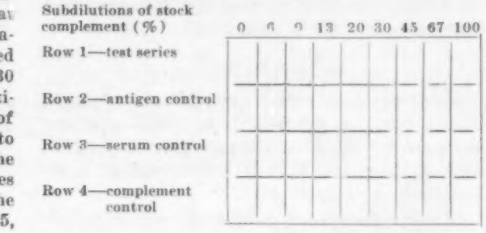
Rabbit serum, in a dilution of 1:40, was used in the antigen dilution method of CF. Each antigen was tested for anticomplementary activity at the lowest dilution (1:40) in the presence of 1, 2, 3, 4, and 5 hemolytic ($C'H_{50}$) units.

Animals were destroyed at various days following infection, and lymph nodes were assayed for CF and agar diffusion activity as shown (table 2).

By substituting one hour fixation at 37 C. for overnight fixation at 5 C., titers were lowered approximately 25 per cent.

Complement Fixation by Complement Dilution (Quantitative).—This procedure has been used extensively in typing foot-and-mouth disease virus.[8] Antigen and antibody concentrations are constant and the complement dilutions are varied.

A stock dilution of complement, usually 1:30, was prepared; subdilutions were made containing 6, 9, 13, 20, 45, and 67 per cent of the stock dilution per unit volume. Each increment consisted of 50 per cent more of the stock dilution of complement than was used for the preceding subdilution. The tube rack plan was as follows:


Subdilutions of stock complement (%)
0 6 9 13 20 30 45 67 100
Row 1—test series
Row 2—antigen control
Row 3—serum control
Row 4—complement control

Each tube contained: (row 1) 1.0 ml. of each complement subdilution, 0.4 ml. of antigen, and 0.4 ml. of serum; (row 2) 1.0 ml. of each complement subdilution, 0.4 ml. of antigen, and 0.4 ml. of VBS; (row 3) 1.0 ml. of each complement subdilution, 0.4 ml. of VBS, and 0.4 ml. of serum; (row 4) 1.0 ml. of each complement subdilution and 0.8 ml. of VBS. In the first vertical row (0%), complement was replaced by VBS and served as a hemolytic control for each reagent.

After incubation in a water bath at 37 C. for one hour, 0.4 ml. of indicator system was added. Incubation was continued for 30 minutes, followed by centrifugation at 1,000 x g for 10 minutes.

The degree of hemolysis was determined in a Beckman DU spectrophotometer at 541 mμ. The percentages of hemolysis were calculated, converted to probits,[5] and plotted as the ordinate. The logarithms of the subdilutions of stock C′ were plotted as the abscissa and the $C'H_{50}$* was determined.

To determine optimal dilutions of antigen and antiserums, antigen dilutions of 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320 were titrated against each of four dilutions of antiserum at dilutions of 1:20, 1:40, 1:60, and 1:80, respectively. The results are shown (fig. 1). Serum at 1:40 was chosen as optimal for maximum fixation and to avoid anticomplementary activity encountered at lower dilutions. An antigen dilution of 1:40 was sufficient to avoid the prozone effect of lower dilutions and adequate to provide sufficient material for maximal fixation of C′.

Lymph nodes from normal cattle and from rinderpest-infected cattle were collected and tested to establish the value of a significant titer. Mesenteric, mandibular, and prescapular lymph nodes from infected cattle were harvested two to six days after the onset of fever, prepared as a 1:40 dilution of antigen, and tested with antiserum diluted 1:40.

The logarithm of C′ for 50 per cent hemolysis was determined for antigen and for antigen with

* Point where the experimental line intercepts the horizontal line of probit 5.00. A perpendicular line to the abscissa represents the logarithm of C′ for 50 per cent hemolysis.

serum and converted to antilogarithms which represent the volumes of C′ for 50 per cent hemolysis. The difference between these volumes is the C′ fixed by the homologous system. The volume of C′ in the presence of antigen is 1 $C'H_{50}$ unit. To determine the number of $C'H_{50}$ units fixed by the immune system, the volume of C′ fixed was divided by 1 $C'H_{50}$ unit. The number of $C'H_{50}$ units fixed in one hour are shown (fig. 2).

RESULTS

Rinderpest-infected mesenteric lymph nodes can be processed to yield specific antigen with an anticomplementary titer of less than 1 $C'H_{50}$ at a dilution of 1:40 (table 1).

Mesenteric lymph nodes harvested at the optimum time of five to seven days postinfection, which corresponds to three to five days of fever, gave antigen titers of 1:100 to 1:400 with one hour fixation and 1:160 to 1:560 with overnight fixation. Normal mesenteric lymph node extracts that were diluted beyond 1:10 did not fix C′.

The duration of extractable CF antigen in mesenteric lymph nodes is shown (table 2). These nodes, collected four to nine days

TABLE 2—Rinderpest Diagnosis: Duration of Complement Fixing Antigen in Mesenteric Lymph Nodes from Infected Steers

Postinfection (days)	Animals tested (No.)	Complement-fixation titer*	Agar diffusion titer
3	1	1:20	1:2
5	17	1:200 to 1:560	1:8 to 1:16
6	1	1:400	1:32
7	3	1:160 to 1:360	1:8 to 1:32
8	3	1:120 to 1:320	1:8 to 1:32
10	2	1:20	Negative
12	1	1:40	Negative
14	1	Negative	Negative
17	1	Negative	Negative

* Overnight fixation at 5 C.

after infection, will yield a sufficient concentration of CF antigen for diagnosis.

The CF activity of different ratios of antigen to antiserum after one hour of fixation is illustrated (fig. 1). At a 1:20 dilution of antiserum, the amount of C′ fixed was proportional to the concentration of antigen. However, with higher dilutions of antiserums, a prozone effect occurred with antigen dilutions of 1:10 and 1:20.

The one-hour CF titers of various lymph nodes are shown in terms of $C'H_{50}$ units (fig. 2). Antigens prepared from extracts of lymph nodes fixed hemolytic units of

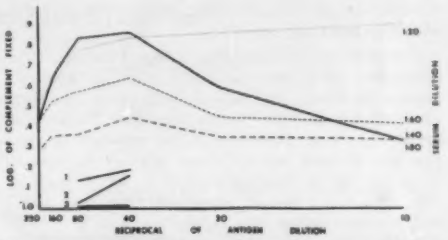


Fig. 1—Complement fixation of rinderpest antigen and antiserum in various ratios. (1) normal tissue replaced infected tissue; (2) normal serum replaced hyperimmune serum; (3) normal tissue in the presence of normal serum.

complement as follows: normal mesenteric lymph nodes, 1 to 3 units; prescapular lymph nodes from infected cattle, 6 units; mandibular lymph nodes from an infected steer that had been dead 12 hours, 10 units; mesenteric lymph nodes from infected cattle, 12 to 19 units.

Freezing and storage of the antigen did not alter its CF activity or ability to produce precipitin lines. Satisfactory results were obtained using nodes collected 12 hours postmortem. At the height of the fever, three to six days postinfection, lymph nodes other than mesenteric were less satisfactory.

DISCUSSION

Several rinderpest-like diseases (mucosal disease, virus diarrhea, malignant catarrhal fever, papular stomatitis,[12] ulcerative stomatitis,[13] nonspecific erosive stomatitis, and gastroenteritis) must be considered in the diagnosis of rinderpest. In rinderpest-infected cattle, mandibular lymph nodes contain adequate antigen when collected during the febrile response. The nodes are sufficiently superficial to permit biopsy if harvest of the preferred mesenteric lymph nodes is not feasible.

The CF titers may appear excessive for viral antigens, but they are not without precedent. Recently, Ross[14] reported CF titers in excess of 1:12,000 with psittacosis virus. Furthermore, due to the extremely destructive effect of the virus on lymphatic tissue and epithelium of the gastrointestinal tract, one could expect to find concentrations of virus in adjacent lymph nodes such as the mesenteric.[15] This may partially account for the relatively low content of antigen in prescapular lymph nodes as

compared to mandibular and to mesenteric lymph nodes which drain the areas of virus activity.[17] White [18] supported this observation when he adapted the agar diffusion test to rinderpest, using mesenteric lymph nodes as antigen. The amount of antigen necessary for detection by this procedure must be relatively high. Foot-and-mouth disease virus in vesicular fluids has been estimated to be in the order of 10^{13} particles per milliliter and does not form visible lines in agar when diluted 1:2.[4] Agar diffusion titers of rinderpest antigen ranged from 1:8 to 1:32, which is an order of magnitude higher than could be obtained from foot-and-mouth disease virus.

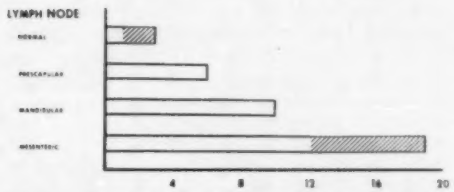


Fig. 2—The hemolytic ($C'H_{50}$, quantitative method) units fixed in one hour by antigens prepared from lymph node extracts. The diagonal lines indicate the range of fixation. The prescapular and mesenteric nodes were collected from infected cattle two to six days after the onset of fever; the mandibular node was from a steer that had been dead for 12 hours.

The relatively rapid and gentle treatment of tissue during extraction by this procedure prevents a significant loss of antigen, since rinderpest virus is susceptible to physical surroundings; *i.e.*, variations in pH, temperature, and dehydration.[8]

Complement fixation can be compared to an antigen-antibody precipitin curve,[10] and the degree of fixation is proportional to the precipitate with three distinct zones: antibody excess, equivalence, and antigen excess. Starting at the antibody excess zone, CF would begin when the immunologic mass has reached a certain minimum size. The amount of C′ fixed would rise to a maximum at or near the equivalence zone, then fall in the zone of antigen excess, becoming asymptotic in the region of extreme antigen excess.

Demonstration of this phenomenon is uncommon with viral antigens and has been reported by Fulton [6] with influenza virus. Brooksby [3] was also able to give an indication of a zone of antigen excess with foot-and-mouth disease virus.

The dilution curves for serum 1:40, 1:60, and 1:80 (fig. 1) are examples of an antigen excess zone which is indicated by a drop in the logarithm of C′ fixed at antigen dilutions of 1:10 and 1:20 as compared to the antigen dilution of 1:40. The serum dilution curve at 1:20 does not show this fall in CF at the more concentrated antigen dilutions, indicating that the serum had not been fully saturated by antigen. The quantitative method of CF as presented (fig. 1) may help to explain some of the stoichiometrical characteristics of antigen-antibody reactions in viral systems.[16]

Summary

A rapid complement-fixation test for the diagnosis of rinderpest is described, using tissue extracts from infected cattle as antigen.

1) Mesenteric, mandibular, and prescapular lymph nodes were collected from cattle four to ten days after infection and up to 12 hours after death. Antigen was extracted from these nodes by high-speed homogenization followed by differential centrifugation.

2) Significant fixation of complement was obtained in one hour, using constant volumes and concentrations of antigen and antiserum and varying dilutions of complement. Significant fixation of complement was not obtained with either normal rabbit serum or noninfected bovine tissue.

3) Antigen prepared from tissues collected five to seven days after infection fixed five 50 per cent hemolytic units of complement in dilutions from 1:100 to 1:400 in one hour and from 1:200 to 1:560 with overnight fixation.

4) High ratios of antigen to antiserum resulted in a prozone phenomenon.

5) Comparable results were obtained using Pendik, Pak Chong (Buffalo), and Kabete strains of rinderpest.

References

[1] Barber, T. L.: Unpublished data, Plum Island Disease Laboratory, Greenport, Long Island, N.Y., 1959.

[2] Boulanger, P.: Application of the Complement-Fixation Test to the Demonstration of Rinderpest Virus in the Tissue of Infected Cattle Using Rabbit Antiserum. I. Results with Kabete and Pendik Strains of Virus. Can. J. Comp. Med. & Vet. Sci., 21, (1957): 379–388.

[3] Brooksby, J. B.: The Technique of Complement Fixation in Foot-and-Mouth Disease Research. A.R.C. Series No. 12, Her Majesty's Stationery Office, London, 1952.

[4] Brown, F., and Crick, J.: Application of Agar Gel Precipitin Tests to Study of the Virus of Foot-and-Mouth Disease. Virology, 5, (1958): 133–143.

[5] Finney, D. J.: Probit Analysis. 2nd ed. The University Press, Cambridge, England, 1952.

[6] Fulton, F., and Dumbell, K. R.: The Serological Comparison of Strains of Influenza Virus. J. Gen. Microbiol., 3, (1949): 97.

[7] Marucci, A. A.: Direct Complement Fixation for Detection of Foot-and-Mouth Disease Antibody in Serums from Experimentally Infected Cattle. Am. J. Vet. Res., 18, (Oct., 1958): 979–984.

[8] Maurer, F.: Rinderpest. XI. The Survival of Rinderpest in Various Mediums. Am. J. Vet Res., 7, (April, 1946): 193–195.

[9] Nakamura, J., and MacLeod, A. J.: The Complement Fixation Test and Its Application to the Diagnosis of Rinderpest. J. Comp. Path., 69, (1959): 11–19.

[10] Osler, A. G., Mayer, M. M., and Heidelburger, M.: Quantitative Studies of Complement Fixation. J. Immunol., 60, (1948): 205–212.

[11] Osler, A. G., Strauss, J. Y., and Mayer, M. M.: Diagnostic Complement Fixation. I. A Method. Am. J. Syph., 36, (1952): 140–153.

[12] Plowright, W., and Ferris, R. D.: Papular Stomatitis of Cattle in Kenya and Nigeria. Vet. Rec., 34, (1959): 718–722.

[13] Pritchard, W. R., Claflin, R. M., Gustafson, D. P., and Ristic, M.: An Infectious Ulcerative Stomatitis of Cattle. J.A.V.M.A., 132, (April 1, 1958): 273–278.

[14] Ross, M. R., and Gogalak, F. M.: The Antigenic Structure of Psittacosis and Feline Pneumonitis Virus. Virology, 3, (1957): 343–364.

[15] Scott, G. R.: The Virus Content of the Tissues of Rabbits Infected with Rinderpest. Brit. Vet. J., 110, (1954): 152–157.

[16] Scott, G. R.: A Precis of the Characteristics of Rinderpest Virus. Bull. Epizoot. Dis. Afr., 1, (1959): 173–178.

[17] Sisson, S., and Grossman, J. D.: The Anatomy of the Domestic Animals. 4th ed. W. B. Saunders Co., Philadelphia, 1953.

[18] White, G.: A Specific Double Diffusible Antigen of Rinderpest Virus Demonstrated by the Agar Double-Diffusion Precipitation Reaction. Nature, 181, (1958): 1409.

SUMMARIO IN INTERLINGUA

Un Rapide Test Serologic pro Peste Bovin

Un rapide test de fixation de complemento pro le diagnose de peste bovin es describite. Le antigeno usate in illo es extractos tissular ab bestial inficite.

1. Nodos lymphatic mesenterie, mandibular, e prescapular esseva colligite ab bestial quatro a dece dies post le infection e usque a 12 horas post morte. Antigeno esseva extrahite ab iste nodos per homogenisation a alte velocitate, sequite per centrifugation differential.

2. Un fixation significative de complemento non esseva obtenite in un hora con le uso de constante volumines e concentrationes de antigeno e antisero e variabile dilutiones de complemento. Un fixation significative de complemento non esseva obtenite con normal sero de conilio o non-inficite tissu bovin.

3. Antigeno preparate ab tissu colligite cinque a septe dies post le infection sufficeva a fixar cinque unitates hemolytic de 50 pro cento de complemento in dilutiones de inter 1:100 e 1:400 in un hora e de inter 1:200 e 1:560 in fixation transnocturne.

4. Alte proportiones de antigeno a antisero resultava in un phenomeno prozonal.

5. Comparabile resultatos esseva obtenite con le uso del racias Pendik, Pak Chong (bufalo), e Kabete del virus de peste bovin.

Clinical and Hematologic Studies of Lymphosarcoma in a Herd of Cattle

G. H. THEILEN, D.V.M.; O. W. SCHALM, D.V.M., Ph.D.;
VIRGINIA GILMORE, B.S.

Davis, California

CLINICAL LYMPHOSARCOMA was diagnosed five times and twice confirmed histopathologically over a five-year period in purebred, closely linebred, dairy cattle in a single herd, offering a unique opportunity to investigate the disease as a herd problem. Two complete clinical and hematologic investigations on 50 cows were conducted, with a 14-month interval between the two studies.

A number of reports describing essentially the terminal phases of bovine lymphosarcoma may be found in the veterinary literature of the United States.[1, 5, 10, 12, 13, 15, 21, 29] Many of the popular breeds of cattle are included in these case studies. Little or no reference is made in the American literature to lymphosarcoma as a herd problem. The foreign literature, however, has several references describing the disease in the herd.[1–3, 6, 7, 16, 24, 26]

Neoplastic disease involving the lymphoid tissues has not been considered of serious economic import in the United States. Nevertheless, this herd study and foreign reports indicate that lymphosarcoma might be of considerable concern in some herds in which this neoplastic disease has been positively diagnosed. In 1958, on the basis of condemnations in federally inspected meat packing establishments, the incidence of bovine lymphosarcoma in the United States was approximately 1.7 cases per 10,000 cattle. Stations reporting the highest percentage of condemnations were in states east of the Rocky Mountains. Reports from all stations in the 11 western states were below the national average. Condemnations in federally inspected establishments during the fiscal years 1945-1958 have more than doubled.[29] The reason for the increase is not fully understood, although more accurate reporting may be responsible in addition to an absolute increase in the disease.

Bovine lymphosarcoma has usually been detected late in the course of the disease through the correlation of clinical, hematologic, and histopathologic changes. The outstanding clinical sign is enlargement of external and internal lymph nodes; however, in some instances, only one superficial node will be enlarged, or possibly the only enlargement will be detected when rectal palpation is performed. In some instances the clinical detection of nodular enlargement is impossible, but there may be other signs that lead to suspicion of this lesion, such as neurologic disturbance due to lymph node encroachment on the spinal cord or regional nerves.[25]

The abomasum and first part of the duodenum are commonly involved by neoplastic growth, frequently causing signs of indigestion and bloating. The myocardium may be invaded, resulting in cardiac signs. Other sites commonly involved are liver, urogenital tract, spleen, lungs, pancreas, adrenals, skeletal muscles, and periorbital tissue.[2, 6] Bendixen[2] has suggested three clinical groups: (1) animals in which the prominent signs are lymph node enlargement, (2) signs in which the internal organs are particularly involved, and (3) signs in which the skin and associated lymph nodes are involved.

In studies at this school, it has been found that either leukopenia or lymphopenia may be consistent with the disease; however, it is common to find total white blood cell counts of 20,000 to 40,000/cmm. and counts as high as 195,000/cmm. have been observed. Prolymphocytes, lymphoblasts, and atypical lymphocytes are frequently observed.

To date, only meager clinical and pathologic correlation has been established between the early hematologic stage and the actual classical disease. However, radioactive thymidine-labeled mononuclear blood cells from some cows in this herd have given additional information.[22] In this report, the term "lymphosarcoma" refers to the classical disease as well as to the early hematologic conditions.

MATERIALS AND METHODS

Jugular blood (about 5 ml.) was drawn into a vial containing dry ammonium and potassium oxalate.[8] Within 30 minutes, blood films were prepared on coverglasses and stained by Wright's method. The vials were placed in the refrigerator and all total leukocyte counts were completed within 24 hours after collection of the blood. All leukocyte counts, total and differential, were made by one of the authors (V. G.).

Received for publication Nov. 10, 1959.

From the School of Veterinary Medicine, University of California, Davis.

These studies were aided in part by a research grant from the University of California Cancer Research Foundation.

The authors thank Otto C. Straub for personal consultations and translations, and Donald L. Dungworth for the pathologic work.

Cells of the lymphocytic series were classified as lymphoblast, prolymphocyte, or lymphocyte, according to the criteria of Schalm.[20] The lymphoblast was generally a large cell with at least one nucleolus distinguishable from the rest of the nucleus by staining a light blue stain; there was more cytoplasm in relation to nucleus than is common in the large, mature lymphocyte. The prolymphocyte was a large cell with considerably more cytoplasm than is common to mature lymphocytes, and the nucleus generally had one or more ring-like structures, representing nucleolar remnants. The mature lymphocyte, though not separated into large and small varieties for counting, had an acentric compact nucleus and a limited cytoplasmic crescent. Generally, the cytoplasm of immature lymphocytes was more basophilic and the nuclear chromatin was more diffuse than that of the more mature lymphocytes. Each differential percentage was based on a count of 200 cells. The present discussion concerns data on 50 cows, 2 to 17 years old, that were present in the herd during investigations.

Normal bovine blood values (table 1)[20] were utilized to establish the upper limit for the absolute total lymphocyte count consistent with a classification of normal; namely, lymphocytes, 9,000/cmm. and total leukocytes, 12,000/cmm. of blood.

TABLE 1—Ranges and Means for Leukocytes in Normal Bovine Blood

Component	Range (No./cmm.)	Mean (No./cmm.)	Range (%)	Mean (%)
Leukocyte	4,000 to 12,000	8,000	.....	.....
Band neutro.	0 to 240	40	0 to 2	0.5
Mature neutro.	600 to 5,400	2,240	15 to 45	28
Lymphoblasts	0	0	0	0
Prolymphocytes	0	0	0	0
Lymphocytes	1,800 to 9,000	4,640	15 to 75	58
Monocytes	80 to 840	320	2 to 7	4
Eosinophils	80 to 2,400	720	2 to 20	9
Basophils	0 to 240	40	0 to 2	0.5

A thorough clinical examination was conducted, including past history, genetic relationship, and performance of the individual. Tissues were also collected from 9 cows which for various reasons were sent to slaughter from this herd. Tissue sections were taken from various external and internal lymph nodes, liver, spleen, heart, abomasum, kidneys, lungs, adrenals, and pituitary and immediately placed into freshly prepared Zenker's solution. The sections were imbedded in paraffin and stained with hematoxylin and eosin.

RESULTS

We have established, as a result of these studies, a tentative hematologic classification of lymphosarcoma-positive, lymphosarcoma-suspect, and probably normal; classification was based on the past history and hematologic studies in this herd as compared with the normal standards established by Schalm.[20] Holman [9] and Moberg [14] have recognized that the normal bovine total leukocyte count drops with advancing age. It was also demonstrated in this study that total leukocyte count of normal cows falls with advancing age, and that the mean absolute lymphocyte number per cubic milliliter of blood was reduced by nearly 50 per cent in cows 5 years of age and over. This suggests that lower total leukocyte and absolute lymphocyte counts may be given greater significance in the diagnosis of lymphosarcoma with advancing age. Therefore, to establish normal leukocyte values in relationship to age in this particular herd and to clarify leukocytic values of cows classified as suspect or positive for lymphosarcoma, the data were tabulated in three age groupings, cows 2 to 5 years old (table 2); cows 5 to 9 years old (table 3); and cows 10 to 17 years old (table 4).

Leukocyte Counts for Probably Normal Cows.—Twenty-seven cows (54% of all cows studied) were classified as having normal leukocyte counts. Fifteen cows (68%) 2 to 5 years old (table 2); 5 cows (38%) 5 to 9 years old (table 3); and 7 cows (46%) 10 to 16 years old (table 4) were classified normal for each respective age group. These data demonstrate that total leukocyte counts up to 12,000/cmm. of blood (table 2) could be considered normal for the younger cows in this herd, though total leukocyte count did not exceed 8,000/cmm. in cows 5 to 16 years old (tables 3, 4) that were classified as having normal leukocyte blood levels.

The range, per cent, and absolute number of lymphocytes per cubic milliliter of blood from cows classified as normal with respect to the lymphosarcoma problem are also presented (tables 2–4). Cells designated as prolymphocytes composed up to 3 per cent of the differential leukocyte count in the blood of cows classified as normal on the basis of total leukocytes and absolute lymphocyte number. Since the presence of lymphoblasts was regarded as evidence of abnormality, no cow for which a single lymphoblast had been recorded was included among the normal animals, even though the total leukocyte and absolute lymphocyte counts were low.

TABLE 2—Total Leukocyte and Differential Lymphocyte Counts in Cows 2 to 5 Years Old

Cow No.	Age (years)	Leukocyte count per cmm. blood	Differential distribution of lymphocyte types (%)			Total lymphocytes	
			Blast cells	Prolymphocytes	Mature lymphocytes	Per cent	Absolute number/cmm.
PROBABLY NORMAL (68.2%)							
1677	2	12,300	0	0	67.0	67.0	8,241
	3	12,100	0	2	51.0	53.0	6,413
1631	2	8,400	0	0	75.5	75.5	6,342
	3	11,200	0	2	65.0	67.0	7,504
1593	2	11,100	0	2	41.5	43.5	4,828
	3	9,050	0	0	56.0	56.0	5,068
1590	3	8,300	0	1	41.5	42.5	3,527
	4	8,500	0	1	40.0	41.0	3,485
1582	3	9,800	0	1	68.5	69.5	6,811
	4	11,700	0	1	52.0	53.0	6,201
1549	3	7,750	0	2	62.5	64.5	4,998
	4	7,650	0	2	40.0	42.0	3,213
1546	3	9,350	0	3	67.0	70.0	6,545
	4	7,750	0	1	59.0	60.0	4,650
1544	3	9,400	0	1	63.5	64.5	6,063
	4	8,600	0	0	34.0	34.0	2,924
1535	3	7,200	0	0	57.5	57.5	4,140
	4	6,150	0	0	53.0	53.0	3,259
1526	3	10,450	0	0	65.0	65.0	6,792
	4	11,850	0	1	68.0	69.0	8,176
1523	3	8,650	0	1	65.0	66.0	5,709
	4	10,800	0	2	56.0	58.0	6,264
1517	3	6,800	0	3	58.0	61.0	4,148
	4	7,200	0	2	54.0	56.0	4,032
A 7	3	9,100	0	2	57.5	59.5	5,414
	4	10,850	0	0	52.0	52.0	5,642
A 6	3	5,400	0	0	79.0	79.0	4,266
	4	9,050	0	0	56.0	56.0	5,068
1511	4	8,500	0	3	75.0	78.0	6,630
	5	7,850	0	0	54.0	54.0	4,239
range		5,400–12,300	0	0–3	34.0–79.0	34.0–79.0	2,924–8,241
mean		(9,093)				(59.0)	(5,353)
LYMPHOSARCOMA-SUSPECT (9.1%)							
1589	2	8,050	0	1	64.0	65.0	5,232
	3	8,200	1*	10*	66.0	77.0	6,313
A 3	4	10,500	0	1	62.0	63.0	6,615
	5	12,100	2*	4*	38.0	44.0	5,324
LYMPHOSARCOMA-POSITIVE (22.6%)							
1577	2	9,200	0	0	58.5	58.5	5,382
	3	22,050	2	30	46.0	78.0	17,199**
1574	3	9,050	0	0	71.0	71.0	6,425
	4	16,500	0	1	64.0	65.0	10,725**
1568	3	14,500	0	2	65.5	67.5	9,787**
	4	10,900	0	0	63.0	63.0	6,867
1542	3	13,400	2	11	60.0	73.0	9,782**
	4	19,750	1	4	85.0	90.0	17,775**
1474	4	12,950	0	1	76.0	77.0	9,971**
	5	11,000	0	1	75.0	76.0	8,360*
range†		12,950–22,050	0–2	1–30	46.0–85.0	65.0–90.0	9,782–17,775
mean		(16,525)				(75.0)	(12,539)

* Leukocyte value suggesting a suspect classification for lymphosarcoma for the particular age group and herd in question. ** Positive blood counts. † Limited to six positive blood samples.

Leukocyte Counts for Lymphosarcoma-Suspect Cows.—A cow was classified as suspect for lymphosarcoma when the absolute lymphocyte count was less than 9,000/cmm. but above the normal maximum established within the herd for the age group in question (cow 1442, table 3); when prolymphocytes constituted 4 per cent or more of the differential leukocyte count (cow 1343, table 3); or when lymphoblasts were recorded as present although the absolute lymphocyte count did not exceed the normal range for the age group (cows A3 and 1589, table 2; cow 1355, table 3; and cow 1010, table 4). On this basis, 6 cows (12%) were classified as suspect for lymphosarcoma, although only two of them (cows 1343 and 1442, table 3) could be so classified by means of the blood examinations, made 14 months apart.

Leukocyte Counts for Lymphosarcoma-Positive Cows.—Seventeen cows (34%)

TABLE 3—Total Leukocyte and Differential Lymphocyte Counts in Cows 5 to 9 Years Old

Cow No.	Age (years)	Leukocyte count per cmm. blood	Differential distribution of lymphocyte types (%)			Total lymphocytes	
			Blast cells	Prolymphocytes	Mature lymphocytes	Per cent	Absolute number/cmm.
PROBABLY NORMAL (38.4%)							
1444	5	6,150	0	0	51.5	51.5	3,167
	6	6,800	0	0	39.0	39.0	2,652
1433	5	7,600	0	0	44.5	44.5	3,382
	6	6,450	0	2	45.0	47.0	3,031
1376	7	5,600	0	2	63.0	65.0	3,640
	8	4,850	0	1	36.0	37.0	1,794
1327	7	6,600	0	2	45.0	47.0	3,102
	8	6,350	0	0	45.0	45.0	2,857
1301	7	6,850	0	0	40.0	40.0	2,740
	8	7,150	0	0	40.0	40.0	2,860
range		4,850–7,600	0	0–2	36.0–63.0	37.0–65.0	1,794–3,382
mean		(6,440)				(46.0)	(2,922)
LYMPHOSARCOMA-SUSPECT (23.1%)							
1442	5	11,950	0	3	60.5	63.5	7,588*
	6	8,650	0	0	65.0	65.0	5,622*
1355	7	5,400	0	0	53.5	53.5	2,889
	8	4,600	1*	1	43.0	45.0	2,070
1343	7	6,900	0	0	52.5	52.5	3,622*
	8	5,700	0	4*	75.0	79.0	4,503
LYMPHOSARCOMA-POSITIVE (38.4%)							
1416	5	19,050	0	10	85.0	95.0	18,097
	6	20,250	0	8	82.0	90.0	18,225
2352	5	12,800	0	3	73.0	76.0	9,728
	6	17,700	0	8	67.0	75.0	13,275
C-68	6	20,200	0	15	68.0	83.0	16,766
	7	18,200	1	3	72.0	76.0	13,832
1352	7	11,000	0	3	92.5	95.5	10,505
	8	17,150	0	22	74.0	96.0	16,464
1278	8	13,950	0	8	74.0	82.0	11,439
	9	18,800	2	10	69.0	81.0	15,228
range		11,000–20,250	0–2	1–22	67.0–92.5	75.0–96.0	9,728–18,225
mean		(16,910)				(81.5)	14,356)

* Leukocytic value suggesting classification as suspect for lymphosarcoma for the particular age group and herd in question.

were classified as positive for lymphosarcoma on the basis of an absolute lymphocyte count in excess of 9,000/cmm. of blood. The data indicated an increase-with-age relationship, as absolute lymphocyte counts were in excess of 9,000/cmm. in one or both blood examinations in 22.6 per cent of cows 2 to 5 years old, 38.4 per cent of cows 5 to 9 years old, and 46.6 per cent of cows 10 to 17 years old.

With respect to total leukocyte counts among the 17 cows classified as positive for lymphosarcoma, only 2 cows had counts in excess of 25,000 leukocytes per cubic milliliter of blood; the remaining 15 cows had total counts between 11,000 and 22,000. Among the 29 blood samples from cows classified as positive for lymphosarcoma, 10 contained lymphoblasts and 17 samples (57%) had prolymphocyte contents of 4 per cent or more.

In the lymphosarcoma-positive cows, the lymphocytes composed 65 to 90 per cent of the differential leukocyte count in cows 2 to 5 years old; 75 to 96 per cent in cows 5 to 9 years old; and 58.5 to 94 per cent in cows 10 to 17 years old. The mean values were 75.0, 81.5, and 79.0 per cent, respectively.

Nine cows changed in classification over the 14-month interval. Of 5 cows classified as normal on the first investigation, 3 (1589, A3, and 1355) became suspect and 2 (1577 and 1574) positive 14 months later. Cow 1010, classified suspect in the first study, was normal the second time, and cow 1035 was positive. Of 2 cows classified as positive in the first investigation, 1 (1568) was normal the second time and the other (1474) became suspect.

Physical Examination of Lymphosarcoma-Positive Cows.—Many cows in the herd were subjected to physical examinations. Six cows classified as positive had distinctly abnormal total leukocyte and absolute lymphocyte counts. Temperature, pulse, and respiration rates were normal, however, and palpation of external lymph

nodes or rectal examination gave no evidence of node enlargement. Four of the 6 cows had subcutaneous swellings; a biopsy from cow 1087 revealed a lipoma on section (table 5). All of these cows were in excellent physical health. The possibility that genetic factors were involved was investigated, but there were no suggestions of simple inheritance.

Histologic Study of Tissues.—Nine cows slaughtered were clinically negative for lymphosarcoma and only 1 was hematologically positive. Neither gross nor histologic examination revealed significant lesions (table 6).

DISCUSSION

This report deals with detection, by means of blood studies, of possible evidence of a malignant disease of lymphocytic tissue in the bovine animal. The term "lymphosarcoma" has been chosen because the blood leukocyte count in this disease is variable and not always diagnostic. Lymphosarcoma has been defined [22] as a malignant tumor of lymphoid tissue that is predominantly lymphocytic or lymphoblastic. It has also been stated: "lymphocytic leukemia is the demonstrable presence of malignant lymphocytes or lymphoblasts in the circulating blood, and that there are no differences between lymphosarcoma and lymphatic leukemia in either gross or microscopic pathology." [31]

Recently, Bendixen [1] and, earlier, Goetze [6] recognized the existence of leukosis-affected (lymphosarcoma) herds, the infection of nonaffected herds by the introduction of animals from leukosis-affected herds, and the possibility of spread of the disease by contact. The (apparently) infectious nature of this disease has led to a control

TABLE 4—Total Leukocyte and Differential Lymphocyte Counts in Cows 10 to 17 Years Old

Cow No.	Age (years)	Leukocyte count per cmm. blood	Differential distribution of lymphocyte types (%)			Total lymphocytes	
			Blast cells	Prolymphocytes	Mature lymphocytes	Per cent	Absolute number/cmm.
PROBABLY NORMAL (46.6%)							
RB4	10	7,200	0	1	53.0	54.0	3,888
	11	7,900	0	0	59.0	59.0	4,661
1194–	12	4,400	0	1	40.0	41.0	1,804
	13	5,300	0	2	43.0	45.0	2,385
1149	12	6,450	0	1	43.0	44.0	2,838
	13	6,400	0	1	53.0	54.0	3,456
1141	12	6,250	0	1	46.0	47.0	2,937
	13	6,400	0	1	42.0	43.0	2,762
1125	12	4,100	0	0	44.0	44.0	1,804
	13	6,100	0	1	43.0	44.0	2,684
983	14	6,200	0	0	37.0	37.0	2,294
	15	6,850	0	1	42.0	43.0	2,945
57	15	6,050	0	2	45.0	47.0	2,843
	16	5,250	0	0	56.0	56.0	2,940
range		5,250–7,900	0	0–2	37.0–59.0	37.0–59.0	1,804–4,661
mean		(6,060)				(47.0)	(2,874)
LYMPHOSARCOMA-SUSPECT (6.6%)							
1010	13	5,050	1*	3	29.0	33.0	1,666
	14	6,950	0	0	42.0	42.0	2,879
LYMPHOSARCOMA-POSITIVE (46.6%)							
1231	10	11,550	0	3	81.0	84.0	9,702
	11	13,600	0	1	82.0	83.0	11,288
1168	12	15,000	1	9	72.5	82.5	12,375
	13	15,250	2	6	67.0	75.0	11,437
1087	13	32,100	3	21	70.0	94.0	30,174
	14	50,200	5	72	17.0	94.0	47,188
1071	13	28,000	0	1	57.5	58.5	16,380
	14	27,800	2	6	59.0	67.0	18,626
1035	14	11,650	0	2	62.0	64.0	7,456*
	15	14,550	0	7	72.0	79.0	11,494
934	14	19,000	0	0	86.0	86.0	16,340
	15	16,050	0	4	75.0	79.0	12,679
881	16	14,350	0	1	72.5	73.5	10,547
	17	17,450	0	2	65.0	67.0	11,691
range**		11,550–50,200	0–5	0–72	17.0–86.0	58.5–94.0	7,456–47,188
mean**		(21,146)				(79.0)	(16,917)

* Leukocytic value suggesting classification as suspect for lymphosarcoma for the particular age group and herd in question. ** Limited to the 13 positive blood samples.

TABLE 5—Results of Physical Examination of Selected Cows Classified as Positive for Lymphosarcoma*

Cow No.	Age (years)	w.b.c./cmm. on day of exam.	Lymphocyte series			Absolute lymphocyte count/cmm.	Temp. pulse resp.	Comments†
			Blast cells	Prolympho-cytes	Mature			
1474	5	16,050	0	7.5	74.5	13,161	101.4 80 28	None.
1352	8	23,450	0	0.0	88.0	20,136	101.0 80 36	None.
1278	9	16,900	0	3.5	79.0	13,942	101.0 88 30	Subcutaneous swelling behind right olecranon; 8 x 8 cm. Double 1st heart sound.
1071	14	22,500	2	15.0	78.5	21,487	100.8 74 24	Subcutaneous swelling over left 8th rib; 5 x 8 cm.
934	15	17,500	0	6.0	56.0	10,850	101.6 80 20	Subcutaneous swelling on right side of thorax; 10 x 15 cm.
1087	14	29,950	0	4.5	74.0	23,170	101.4 70 20	Subcutaneous swelling on escutcheon; 3 x 5 cm. Biopsied lipoma.

* Blood study, day of physical examination. † Lymph nodes were not enlarged in any of these cows.

program in Denmark, where the disease is now reported.[3] In addition, Papparella[18] has obtained a virus from bovine lymphosarcoma tissue, passed it serially in embryonating chicken eggs, and reproduced leukemic signs in guinea pigs and mice. He has obtained electronmicrographs of the virus which was isolated from chicken embryo and found it to measure 135 mμ in diameter.[19]

Goetze *et al.*[7] suggested that all cows in leukosis-affected herds may be classified by a key that employs total leukocyte count and lymphocytes in percentage, a normal blood count consisting of 10,000 or fewer total leukocytes and 60 per cent or less lymphocytes, the blood of a suspect having 10,000 to 18,000 leukocytes per cubic milliliter and 60 to 75 per cent lymphocytes, and a positive sample for lymphosarcoma is one containing total leukocytes in excess of 18,000 per cubic milliliter and lymphocytes above 75 per cent. Niepage[17] has expressed doubt as to the value of such a key. Bendixen[3,26] also suggests that cows may be classified for leukosis (lymphosarcoma) by use of a hematologic key. Bendixen[3] defines a leukosis-affected herd as a herd in which more than 1 bovine animal has been diagnosed as having clinical leukosis. The method has been applied to 59 Danish herds; 36 herds had a past history of more than 1 animal clinically diagnosed with leukosis and the other 23 herds had a history of leukosis being diagnosed in only 1 animal. Thirty-five to 75 per cent of the animals in the 36 herds were classified as positive for leukosis, while hematologic classification of animals in the 23 herds were similar to herds classified as normal, in which the disease had never been clinically recognized. The tentative hematologic classification of 34 per cent lymphosarcoma-positive, 12 per cent suspects, and 54 per cent probably normal in this herd is in close agreement with the findings of Goetze[7] and Bendixen.[3,26]

Thompson *et al.*[29] and Niepage[17] have stressed the importance to diagnosis of the presence of immature lymphocytes in the circulating blood. In this study, most of the cows classified as positive for lymphosarcoma had varying numbers of prolymphocytes, and a few also had lymphoblasts. However, the most consistent changes in cows designated as positive were total leukocyte counts above 12,000/cmm. and absolute total lymphocyte counts in excess of 9,000/cmm.

Lymphosarcoma is said to be seen more commonly in animals 5 years old or more.[17] This was true in this herd, insofar as the blood counts are concerned, for lymphosarcoma was diagnosed in 22.6 per cent of cows 2 to 5 years old, 38.4 per cent of cows 5 to 9 years old, and 46.6 per cent of cows 10 to 17 years.

Goetze *et al.*[7] stated that bovine leukosis

TABLE 6—Histologic Study of Tissues Collected from 9 Cows Sent to Slaughter

Classification for lymphosarcoma	Cow No.	Age (years)	Leukocyte count per cmm. blood	Differential distribution of lymphocyte types (%)			Gross pathology	Histopathology
				Blast cells	Prolymphocytes	Mature lymphocytes		
Normal	1673	3	7,100	0	3	55.0	Leg tumor.	Neurofibroma.
Normal	1649	3	6,400	0	0	58.5	.....*	.....*
Normal	1537	4	10,450	0	0	64.0	Periovarian cyst.	Ovary not sectioned.*
Normal	1125**	13	6,100	0	1	43.0	.....*	.....*
Normal	1010	14	8,450	0	0	27.0	Kidney cysts.	Kidneys: protein leakage into tubules, small scattered accumulations of lymphocytes and plasma cells in the cortical interstitium. Lymph nodes: no evidence of neoplasia. R.E. elements prominent in medullary region.
								Small intestine: large numbers of eosinophils in the lamina propria.
Suspect	1637**	3	10,600	1	3	56.0	.....*	.....*
Suspect	1343**	8	5,700	0	4	75.0	.....*	.....*
Suspect	1121	13	11,500	0	0	56.6	Liver: friable; adrenals: medulla enlarged; kidneys: cysts.	Kidneys: minor lesions of chronic interstitial nephritis. Supermammary, prescapular, and iliac lymph nodes: moderate numbers of eosinophils, variable degree of reticuloendothelial hyperplasia.
Positive	1416	6	16,860	0	0	79.5	Subcutaneous fat tumors; liver: friable; spleen: enlarged.	Lipomas. Spleen: prominent malpighian corpuscles.

* Normal finding. ** Hematology prior to slaughter; all others, day of slaughter.

(lymphosarcoma) has a so-called hyperplastic lymphocytic prestage that can be determined by blood examination. Cattle may remain in prestage for a short period or for several months, years, or even a lifetime, but at any time the prestage may change into a fatal tumorous form in a low percentage of the leukemic animals. This appears to be the situation in the herd we studied, for the cows designated as positive for lymphosarcoma, based on blood leukocyte counts, appeared clinically quite normal. Histologic study during prestage might also reveal normal findings (table 6).

Further investigations are needed to determine the factors responsible for the preclinical hematologic stage of the disease. From Papparella's [18,19] findings it appears possible that the preclinical hematologic state is due to a viral infection. The apparent infectious nature of this disease has been reported by European investigators.[1-3,6,7] In unpublished reports from California,[27] it has been found that clinical lymphosarcoma may be frequently diagnosed in certain herds and that many animals in these herds will have preclinical hematologic changes. Thus, these findings also suggest that bovine lymphosarcoma may be an infectious disease.

Goetze *et al.*[7] also reports a higher incidence of leukosis following parturition, which suggests that the stress of parturition may trigger the hematologic preclinical stage into greater activity, leading to a terminal phase. Stress factors may induce lymphosarcoma, and other factors may be responsible for temporary spontaneous remission.[28] Further investigations are also needed to determine the factors which are responsible for the onset of clinical signs.

This herd study shows it to be possible that a high incidence of lymphosarcoma may be found in an apparently healthy herd of closely related cattle. These findings also suggest that there might be a high incidence of this disease in the United States.

Summary and Conclusions

High incidence of clinical bovine lymphosarcoma observed in recent years in a herd of purebred dairy cattle led to a study of this problem. Twice, at a 14-month interval, the entire herd was studied hematologically and observed clinically. Cows were allotted to three age groups with the following hematologic classification:

1) Of 22 cows 2 to 5 years of age, 5 (22.6%) were hematologically positive for lymphosarcoma; 2 (9.1%) were suspect.

2) Of 13 cows 5 to 9 years of age, 5 (38.4%) were hematologically positive for lymphosarcoma and 3 (23.1%) were suspect.

3) Of 15 cows 10 to 17 years of age, 7 (46.6%) were hematologically positive for lymphosarcoma and 1 (6.6%) was suspect.

Hematologic classification for the entire herd was 34 per cent positive, 12 per cent suspect, and 54 per cent probably normal.

These findings suggest that blood studies may be valuable in determining a high incidence of preclinical hematologic changes in an apparently healthy herd of cattle in which lymphosarcoma has frequently been diagnosed.

References

[1] Bendixen, Hans Jorgen: Undersogelser over Kvaegets Leukose (Studies of Leukosis in Cattle). 1. Kvaegleukosens Forekomst og Udbreddelse i Denmark (The Occurrence and Distribution of Cattle Leukosis in Denmark). Nord. Vet.-med., 9, (1957): 1–33.

[2] Bendixen, Hans Jorgen: Undersogelser over Kvaegets Leukose (Studies of Leukosis in Cattle). 2. Kvaegleukosens kliniske Diagnostik (The Clinical Diagnosis of Leukosis in Cattle). Nord. Vet.-med., 10, (1958): 273–301.

[3] Bendixen, Hans Jorgen: Undersogelser over Kvaegets Leukose (Studies of Leukosis in Cattle). 3. Om Kontrollen med Leukosebesaetninger ved haematologiske Undersogelser (Control of Leukosis Herds Using Hematological Examinations). Nord. Vet.-med., 11, (1959): 733–758.

[4] Boyd, W. L., Karson, A. G., Jasper, D. E., Sellers, A. F., and Collier, J. R.: Leukemic Lymphoblastoma in a Cow with Involvement of the Udder. Am. J. Vet. Res., 8, (Oct., 1947): 330–337.

[5] Feldman, W. H.: Lymphosarcoma of the Bovine Abomasum. J.A.V.M.A., 73, (June, 1928): 206.

[6] Goetze, R., Rosenberger, G., Ziegenhagen, G.: Die Leukose des Rindes. Mhefte f. Vet.-med., 9, (1954): 517–526.

[7] Goetze, R., Ziegenhagen, G., Merkt, H.: Zur Diagnose der Leukose des Rindes. Mhefte f. Tierheilk., 5, (1953): 201–211.

[8] Heller, V. G., and Paul, H.: Changes in Cell Volume Produced by Varying Concentrations of Different Anticoagulants. J. Lab. & Clin. Med., 19, (1934): 777.

[9] Holman, H. H.: The Blood Picture of the Cow. Brit. Vet. J., 111, (1955): 440–457.

[10] Jasper, D. E., Sautter, J. H., and Malmquist, W. A.: Lymphocytoma (Leukemia) in a Cow. J.A.V.M.A., 108, (May, 1946): 334–338.

[11] Kaneko, J. J., Tyler, W. S., Theilen, G. H., and Schalm, O. W.: Autoradiographic Studies of Normal and Leukemic Bovine Leukocytes. Am. J. Vet. Res., 21, (March, 1960): 230–232.

[12] McGown, M. N.: Lymphocytoma in a Milk Cow. Vet. Med., 41, (1946): 254.

[13] Mitchell, K. P.: Lymphocytosis in a Cow. Vet. Med., 38, (1943): 493.

[14] Moberg, R.: The White Blood Picture in Sexually Mature Female Cattle with Special Reference to Sexual Conditions. A Clinical and Experimental Study. Thesis, Stockholm, Sweden, 1955.

[15] Moles, C. E.: Lymphocytomatosis in a Jersey Bull. Vet. Med., 42, (1947): 267.

[16] Niepage, H.: Zur Diagnose der Rinderleukose durch den Leukoseschluessel. Berl. u. Münchener tierärztl. Wchnschr., 67, (1954): 253–255.

[17] Niepage, H.: Erhebungen über die Rinderleukose und Untersuchungen des Blutbildes. Mhefte f. Vet.-med., 8, (1953): 21–25.

[18] Papparella, V.: Contribution à l'étiologie virale de la Leucémie Lymphatique (Contribution as to a Viral Etiology of Bovine Lymphatic Leukemia). International Veterinary Congress, 2, (1959): 157.

[19] Papparella, V.: Il Virus della Leucemia Linfatica dei Bovine Visto al Microscopio Elettranico. Zooprofilassi, 4, (April, 1959).

[20] Schalm, O. W.: A Syllabus on Veterinary Hematology. Published by the author, 1959.

[21] Sellers, A. F., Boyd, W. L., and Karlson, A. G.: Leukemia of the Bovine. Cornell Vet., 34, (1944): 366–369.

[22] Smith, H. A., and Jones, T. C.: Veterinary Pathology. (Chapter 7), Lea & Febiger, Philadelphia (1957): 157.

[23] Stasy, J., and Feldman, W. H.: Leukemia, Lymphoblastoma in a Calf. Am. J. Cancer, 34, (1938): 240–247.

[24] Straub, O. C.: Ueber das Blutbild von gesunden Kaelbern leukosefreier Herkunft in den ersten drei Lebensmonaten und vergleichende Untersuchungen an gleichaltrigen, klinisch gesunden Kaelbern aus Leukosebestaenden. Dissertation, Hannover, 1956.

[25] Straub, O. C., Olander, H. T., and Theilen, G. H.: A Case Report of Lymphosarcoma in a Cow with Vertebral Involvement. Cornell Vet., 50, (1960): 251–258.

[26] Steere, J. L.: Lymphocytic Bovine Leukemia (Leukosis). Mod. Vet. Pract., 40, (1959): 30–37.

[27] Theilen, G. H.: Present Concepts Concerning Bovine Lymphosarcoma in the Herd. Presented at the A.V.M.A. Convention, Denver, August 16, 1960.

[28] Theilen, G. H., Dungworth, D. L., and Straub, O. C.: Bovine Lymphosarcoma. Temporary Spontaneous Remission in a Cow. Cornell Vet., (1960): In press.

[29] Thompson, W. W., and Roderick, L. M.: The Relation of Leukemia and Bovine Lymphocytoma. Am. J. Vet. Res., 3, (April, 1942): 159–165.

[30] United States Department of Agriculture, Agricultural Research Service Meat Inspection Division: Malignant Lymphoma Condemnations in Federally Inspected Establishments During Fiscal Years 1945–1958. Personal communication.

[31] Willis, R. A.: Pathology of Tumors. (chapter 49), C. V. Mosby Co., St. Louis, Mo., (1953): 765.

SUMMARIO IN INTERLINGUA

Investigation Clinic e Hematologic de un Occurrentia de Lymphosarcoma con Alte Incidentia in un Grege de Bestial

Un alte incidentia de clinic lymphosarcoma bovin, observate in recente annos in un grege de bestial de lacteria de racia pur, stimulava un studio de iste problema. Duo vices—con un intervallo de 14 menses—le grege complete esseva examinate hematologicamente e

observate clinicamente. Le vaccas esseva dividite in tres gruppos de etate con le sequente classification hematologic:

1. Inter 22 vaccas de etates de inter 2 e 5 annos, 5 (o 22,6%) esseva hematologicamente positive pro lymphosarcoma, e 2 (o 9,1%) esseva suspecte.

2. Inter 13 vaccas de etates de inter 5 e 9 annos, 5 (o 38,4%) esseva hematologicamente positive pro lymphosarcoma, e 3 (o 23,1%) esseva suspecte.

3. Inter 15 vaccas de etates de inter 10 e 17 annos, 7 (o (46,6%) esseva hematologicamente positive pro lymphosarcoma, e 1 (o 6,6%) esseva suspecte.

Le classification hematologic pro le grege total esseva 34 pro cento positive, 12 pro cento suspecte, e 54 pro cento probabilemente normal.

Iste constatationes suggere que studios hematologic pote esser de valor in determinar un alte incidentia de preclinic alterationes hematologic in un apparentemente salubre grege de bestial in le qual lymphosarcoma ha frequentemente essite diagnosticate.

The Effect of Diethylstilbestrol and Progesterone on the Growth of Four Mastitis-Producing Bacteria

NORMA A. FRANK, M.S., and W. D. POUNDEN, D.V.M., Ph.D.

Wooster, Ohio

FRESHLY CUT legume-grass forage may stimulate an increase in the occurrence of mastitis in dairy cows.[11,12] A possible explanation is that the plant estrogenic substances could be involved because these materials have been found present in variable quantities in such forages.[1,3,10,16] It further has been shown that administration of estrogenic substances to cows may simulate chronic mastitis by causing production of abnormal udder secretions.[2,13]

Estrogenic substances may be in udder secretions, although usually in limited quantities.[4,8,9,17] These substances have been observed to influence bacterial growth. For example, a bacteriostatic effect of diethylstilbestrol on several microorganisms has been demonstrated[14]; the consistent bacteriostatic effect of diethylstilbestrol on micrococci suggested its use as a quantitative measure of this material[7]; the oxidation of several carbohydrates by *Aerobacter aerogenes* was observed to be inhibited by stilbestrol[5]; and stilbestrol appeared to have a stimulatory effect on the growth of *Pseudomonas aeruginosa* in rumen contents.[6]

The means by which microorganisms produce attacks of mastitis appear to be complex[15] but, in general, increased growth and activity of the organisms in udder secretions are of major importance. It seemed desirable, therefore, to investigate *in vitro* the effects of diethylstilbestrol and progesterone on the growth of four mastitis-causing bacteria.

METHODS

The products available were diethylstilbestrol U.S.P. in powder form and an aqueous solution of 17 *alpha* acetoxyprogesterone.* The four organisms used had been isolated from udders during attacks of mastitis. They were: (1) *Streptococcus agalactiae;* (2) *Staphylococcus aureus,* that produced relatively wide zones of *alpha* and *beta* hemolysis; (3) *Escherichia coli;* and (4) a Pseudomonas organism having the characteristics of *Pseudomonas aeruginosa.*

The method followed was similar to that used by Hanka and Lockhart.[7] A stock solution was made from each of the hormones by adding 50 mg. of hormone to 100 ml. of 95 per cent ethyl alcohol. A second stock solution was made by adding 40 ml. of the first solution to 160 ml. of 95 per cent ethyl alcohol; a third by adding 120 ml. of the first solution to 80 ml. of 95 per cent ethyl alcohol. These second and third stock solutions were further diluted with various proportions of sterile distilled water. The proportions chosen permitted adding 1-ml. quantities of respective hormone solution to each 9 ml. of nutrient broth to provide final concentrations ranging from 1.0 μg./ml. to 5.0 μg./ml. in steps of 0.5 μg. Concentrations from 1.0 μg./ml. to 3.0 μg./ml. were prepared from the second solution. The higher concentrations ranging up to 5.0 μg./ml. were prepared from the third stock solution. Concentrations of 3.0 μg./ml. were prepared from the second and third stock solutions, thus giving two sets of readings at this point. To prepare solutions that provided final concentrations in the broth ranging from 0.1 μg./ml. to 0.9 μg./ml. in steps of 0.1 μg./ml., proportions of the solution used to prepare the 0.1 μg./ml. concentration were added to the nutrient broth.

To prepare each inoculum, the organism was grown for 24 hours in nutrient broth at 37 C. The cultures were added in 0.1-ml. quantities to each of the tubes of broth containing the different levels of hormone. Following incubation for 24 hours, the turbidity was read on an Evelyn photoelectric colorimeter using a 660-unit filter.

To conduct the experiments with various combinations of the two hormones, dilutions similar to those made with the single hormones were used.

RESULTS

The relative average growth obtained for each of the bacteria in the presence of the various concentrations of stilbestrol and progesterone was determined by the percentage of light transmitted (fig. 1). The averages are based on the results of six to ten trials.

The results obtained for *Str. agalactiae* showed a limited stimulatory effect in the lower concentrations of both hormones,

Received for publication Dec. 14, 1959.

From the Department of Veterinary Science, Ohio Agricultural Experiment Station, Wooster.

* Provided by Dr. J. O. Reed, Syntex Animal Products, New York 16, N.Y.

particularly in the lowest one; it gradually decreased as the concentrations increased. There was a gradual increase in light transmission as concentrations of progesterone were increased, with the result that from the 2.0 μg./ml. level upward, inhibition of growth was obtained. For stilbestrol, there was sufficiently marked increase in light transmission to indicate a definite inhibition of growth between the 2.0 μg./ml. and the 2.5 μg./ml. levels. This was followed by a more gradual increase. The results for the two preparations of the 3.0 μg./ml. level which had been prepared from different stock solutions are given because they did not synchronize. Regardless of which 3.0 μg./ml. point is used in the projection, the foregoing results can be interpreted in the same way. It should be recognized that in figure 1 the lower concentrations of the hormones have been plotted over a proportionately wider space than the higher ones.

The Staphylococcus, following an apparent stimulation in the 0.1 μg./ml. con-

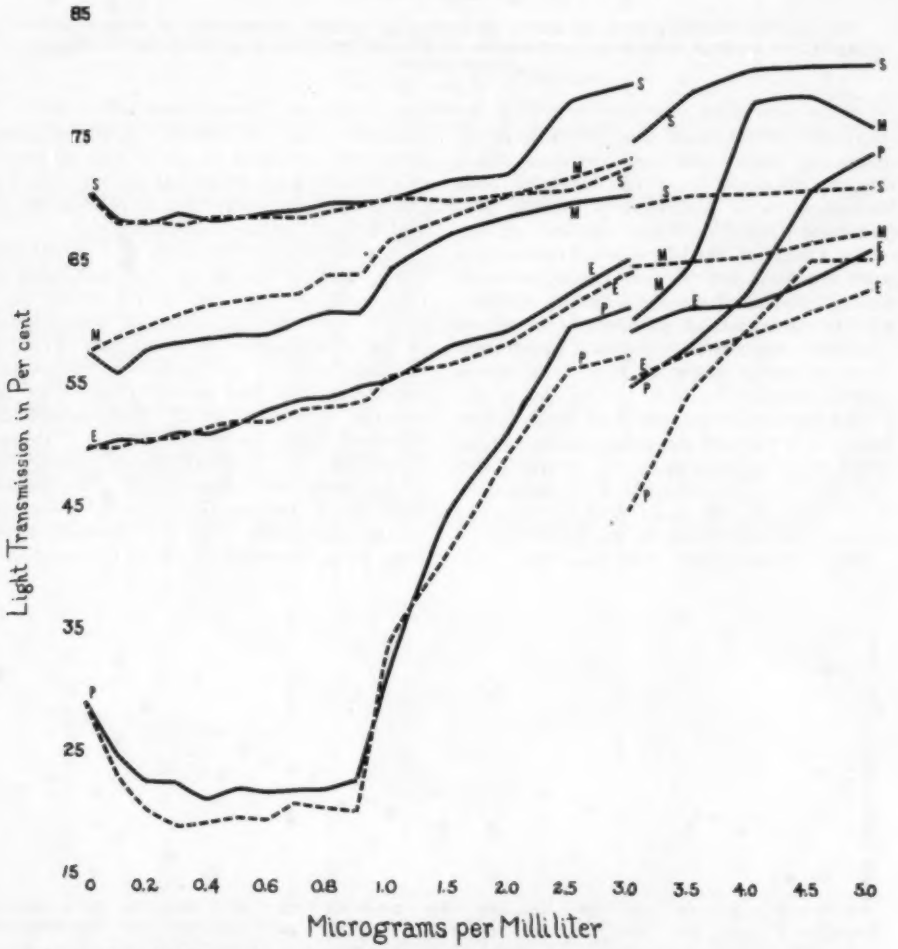


Fig. 1—The relative growth, as shown by percentage of light transmitted, of four mastitis-causing bacteria in broth containing varying quantities of diethylstilbestrol and progesterone. Unbroken lines indicate stilbestrol, broken lines indicate progesterone; S = Streptococcus agalactiae, M = Staphylococcus aureus, E = Escherichia coli, P = Pseudomoas aeruginosa.

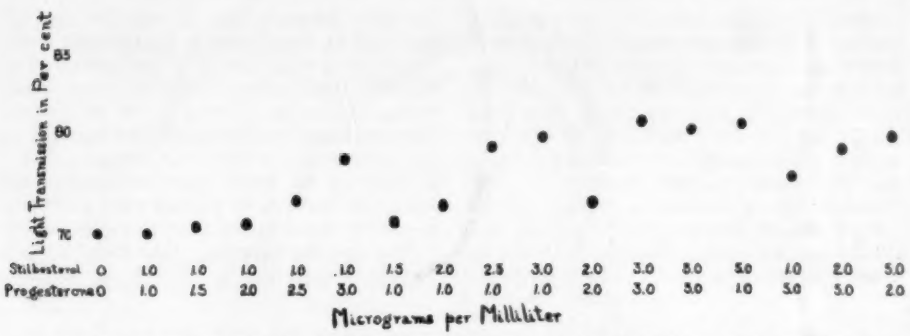

Light Transmission in Per cent
85
80
70
Stilbesterol 0 1.0 1.0 1.0 1.0 1.0 1.5 2.0 2.5 3.0 2.0 3.0 3.0 5.0 1.0 2.0 5.0
Progesterone 0 1.0 1.5 2.0 2.5 3.0 1.0 1.0 1.0 1.0 2.0 3.0 5.0 1.0 5.0 5.0 2.0
Micrograms per Milliliter

Fig. 2—The relative growth, as shown by percentage of light transmitted, of Streptococcus agalactiae in broth containing combinations of diethylstilbestrol and progesterone in varying concentrations.

centration of estrogen, was inhibited in an increasing manner in concentrations above this, up to the 3.0 μg./ml. level by both hormones, more particularly by the progesterone (fig. 1). From the 3.0 μg./ml. to the 4.0 μg./ml. levels, marked inhibitions were recorded for the estrogenic hormone but not the progesterone. At the 5.0 μg./ml. concentration of stilbestrol, there was apparent inconsistency in that less inhibition occurred than at the next lower concentration.

The *Esch. coli* was inhibited by both hormones in a manner somewhat similar to the effect of progesterone on the Staphylococcus (fig. 1). This amounted to increasing inhibition in the mediums containing greater concentrations of the hormones.

The Pseudomonas pattern was quite different from the others. A stimulatory effect was obtained in all of the dilutions of both hormones below 1.0 μg./ml. (fig. 1). Concentrations above this level resulted in well-defined, graded inhibitions.

A second series of tests was conducted using combinations of the two hormones in varying concentrations of 1.0 to 5.0 μg./ml., to determine if either a neutralizing or an enhancing effect occurred. The results obtained for *Str. agalactiae* were somewhat variable but nevertheless were in general agreement with what might be expected from the results of the previous trials (fig. 2). Neutralizing or synergistic effects were not in evidence. The results with *Staph. aureus* also followed those obtained previously (fig. 3). Marked variations from those due to use of the hormones

Light Transmission in Per cent
80
70
60
Stilbesterol 0 1.0 1.0 1.0 1.0 1.0 1.5 2.0 2.5 3.0 2.0 3.0 5.0 5.0 1.0 2.0 5.0
Progesterone 0 1.0 1.5 2.0 2.5 3.0 1.0 1.0 1.0 1.0 2.0 3.0 5.0 1.0 5.0 5.0 2.0
Micrograms per Milliliter

Fig. 3—The relative growth, as shown by percentage of light transmitted, of Staphylococcus aureus in broth containing combinations of diethylstilbestrol and progesterone in varying concentrations.

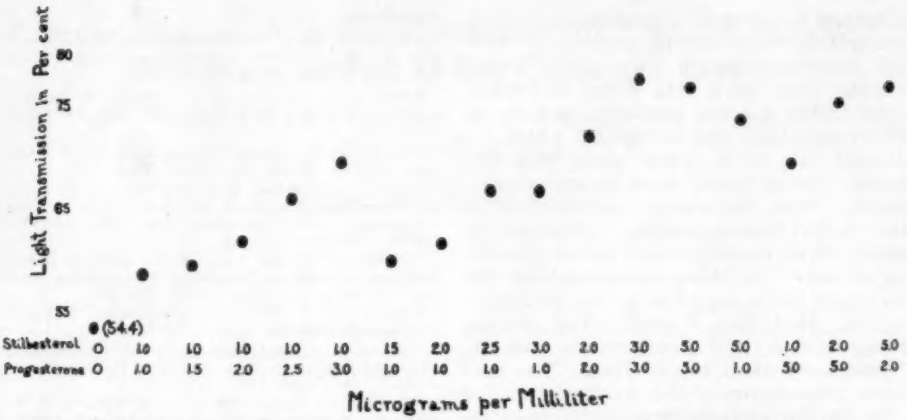


Fig. 4—The relative growth, as shown by percentage of light transmitted, of Escherichia coli in broth containing combinations of diethylstilbestrol and progesterone in varying concentrations.

in combination were not noticeable. Inhibition of some intensity again occurred when concentrations of stilbestrol were above 3.0 μg./ml. The coliform results using the combined hormones had trends similar to those obtained with the single hormones (fig. 4). The hormones were more equally inhibitory on the growth of this organism than on any of the other three in both series of tests.

When Pseudomonas was grown in the higher concentrations of the combined hormones, an additive effect (fig. 5) with marked inhibition was apparent, just as when the hormones were used singly.

DISCUSSION

The finding that diethylstilbestrol was inhibitory to bacterial growth is in agreement with others.[7,14] The rather abrupt increase in inhibition of the Staphylococcus at levels above 3.5 μg./ml. occurred at a somewhat lower concentration than the similar action reported by others to occur above 5.0 μg./ml.[7] An unexpected observation was the intensity of the inhibitory nature of progesterone.

On the basis of the results obtained, it was difficult to see how increased quantities of hormones in udder secretions could

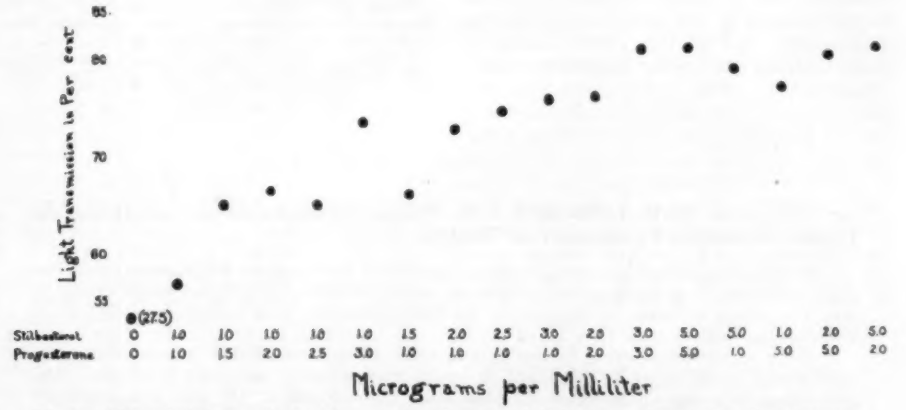


Fig. 5—The relative growth, as shown by percentage of light transmitted, of a Pseudomonas aeruginosa in broth containing combinations of diethylstilbestrol and progesterone in varying concentrations.

stimulate the growth of mastitis-producing bacteria in them, except possibly in very low concentrations.

After this work was done, additional information on the estrogen content of udder secretions was published[4] which indicated this to be much lower than the lowest concentration used in our experiments. Thus, the stimulation observed in the lowest concentrations, although of minor effect, possibly might assume greater significance. In these concentrations, the estrogens had some effect on the Staphylococcus. Both had a slight effect on the Streptococcus; the Pseudomonas was affected more than any of them. The possible relationship of this kind of stimulation to the clinical problem of mastitis associated with Pseudomonas, at a time when cows were on legume pasture, has been considered, but the study is incomplete.

SUMMARY

Four microorganisms, *Streptococcus agalactiae*, a hemolytic *Staphylococcus aureus*, *Escherichia coli*, and a *Pseudomonas aeruginosa*, obtained from udders affected with mastitis, were grown in the presence of diethylstilbestrol and progesterone in concentrations ranging between 0.1 μg./ml. and 5.0 μg./ml.

Both hormones and combinations of them produced inhibition of growth as measured by percentage of light transmitted through the cultures, except in some of the lower concentrations. Slight stimulation of the Streptococcus was observed in the lowest concentrations of both hormones, of the Staphylococcus in the lowest concentration of estrogen, and of the Pseudomonas in concentrations of both hormones below 1.0 μg./ml.

References

[1] Barlett, S., Folley, S. J., and Rowland, S. J.: Oestrogens in Grass and Their Possible Effects on Milk Secretion. Nature, 162, (Nov., 1948): 845.

[2] Berger, J., and Francis, J.: Mastitis in Practice. I. Epizootiology and Bacteriology. Vet. Rec., 63, (April, 1951): 283.

[3] Bichoff, E. M.: Recent Work on Estrogens in Plants. Rep. 5th Tech. Alfalfa Conf., Aug., 1958.

[4] Bitman, Joel, Wrenn, T. R., and Sykes, J. F.: Estrogen Determination in Blood and Body Fluids of Cattle. 3rd Symp. (1957) on Reproduction and Infertility. (1958): 141–156.

[5] Durham, N. N., and Perry, M. D.: Effect of Diethylstilbestrol on Substrate Metabolism in *Aerobacter aerogenes*. J. Bact., 77, (April, 1959): 429–438.

[6] Garner, J. B.: Personal communication, University of Missouri, Columbia, 1959.

[7] Hanka, L. J., and Lockhart, W. R.: Antimicrobial Action as a Basis for Measurement of Diethylstilbestrol. J. Bact., 75, (April, 1958): 471–473.

[8] Lawson, W., Straund, S. W., and Williams, P. C.: Oestrogen Excretion in Milk from Oestrogenized Cattle. J. Endocrinol., 4, (1944–1946): 83.

[9] Munch, Udo: Die Ausscheidung von Naturlichen Androgen und Oestrogen in der Milch. Milch-wissenschaft, 9, (1954): 150.

[10] Pieterse, P. J. S., and Andrews, F. N.: The Estrogenic Activity of Legume Grass and Corn Silage. J. Dai. Sci., 39, (Jan., 1956): 81–89.

[11] Pounden, W. D., Pratt, A. D., Frank, Norma A., and Smith, H. R.: The Incidence of Mastitis in Cows Fed Legume-Grass Mixtures in Fresh Cut Crop and as Silage. J.A.V.M.A., 132, (April, 1958): 337–339.

[12] Pounden, W. D., Frank, N. A., and Vandersall, J. H.: Further Observations on Mastitis in Cows Fed Legume-Grass Forage as Soilage and Silage. J.A.V.M.A., 137, (July 1, 1960): 53–57.

[13] Reece, R. P., and Murphy, J. M.: The Influence of Diethylstilbestrol Dipropionate on the Lactating Mammary Gland of the Cow. J. Dai. Sci., 26, (1943): 748–749.

[14] San Clemente, C. L., and MacKenzie, R. D.: The Bacteriostatic Effect of Diethylstilbestrol. Quart. Bull. Michigan Agric. Exper. Sta., 39, (Feb., 1957): 438–442.

[15] Sanger, V. L., Frank, N. A., and Pounden, W. D.: Udder Reactions to Sterilized Cultures and Filtrates. Am. J. Vet. Res., 20, (July, 1959): 718–722.

[16] Stob, M., Walker, B. J., and Andrews, F. N.: Factors Affecting the Estrogenic Content of Alfalfa Silage. J. Dai. Sci., 41, (March, 1958): 438–439.

[17] Turner, C. W.: Estrogen Content of Colostrum and Milk of Dairy Cattle. J. Dai. Sci., 41, (May, 1958): 630.

SUMMARIO IN INTERLINGUA

Le Effecto de Diethylstilbestrol e de Progesterona Super le Crescentia de Quatro Bacterios Productori de Mastitis

Quatro micro-organismos—*Streptococcus agalactiae*, un hemolytic *Staphylococcus aureus*, *Escherichia coli*, e un *Pseudomonas aeruginosa*, omnes obtenite ab uberes que esseva afficite de mastitis—esseva culturate in le presentia de diethylstilbestrol e de progesterona in concentrationes que variava inter 0,1 e 5,0 μg per ml.

Ambe le hormones—sol e in combination—effectuava un inhibition crescential secundo estimationes basate super le procentage de lumine transmittite a transverso le culturas. Isto non valeva in le caso de alicunes del plus basse concentrationes. Un leve stimulation del streptococcos esseva observate in le concentrationes le plus basse del duo hormones, del staphylococcos in le concentration le plus basse del estrogeno, e del pseudomonades in concentrationes de infra 1,0 μg per ml in le caso de ambe le hormones.

Neural Mechanisms in Sexual Behavior. III. Preputial Muscles and Their Reflexes in Bull Calves and Rams

L. L. LARSON, D.V.M., M.S., Ph.D.; R. L. KITCHELL, D.V.M., Ph.D.; BERRY CAMPBELL, Ph.D.

St. Paul, Minnesota

In the clinical application of the pudendel nerve block technique described previously,[8] difficulty has been experienced in protracting the penis in certain bulls. The difficulty was due principally to a constriction of the preputial orifice and was not due to the activity of the retractor penis muscles. This prompted an investigation of the nerve supply to the preputial orifice, its associated muscles, and the pathways concerned with reflexes involving these muscles. The purpose of this report is to describe the results of this study. Gross anatomic details relative to the nerve supply to the external genitalia have been reported elsewhere.[9]

Review of Literature

The abdominal cutaneous muscle, of which the protractor and retractor preputial muscles are a part, has been well described in many mammals. Its nerve supply has been described as arising from a branch of the anterior thoracic nerve.[1, 7, 10, 14, 17] Other authors use different descriptive terms for its nerve supply, such as pectoral nerves,[2] external thoracic nerve (nervus thoracalis lateralis),[12, 16] subcutaneous thoracic branch,[3] and nervus thoracolateralis of the nervi thoracici caudales.[4, 12]

The protractor and retractor preputial muscles have not been classified with the cutaneous musculature in veterinary anatomy textbooks but are described along with the external genitalia.[11, 16] In no instance is the specific nerve to these muscles mentioned.

Received for publication Jan. 15, 1960.

From the Divisions of Veterinary Obstetrics (Larson) and Veterinary Anatomy (Kitchell), College of Veterinary Medicine, and the Department of Anatomy (Campbell), Medical School, University of Minnesota, St. Paul.

Approved for publication as Paper No. 4298, Scientific Journal Series, Minnesota Agricultural Experiment Station. Dr. Larson is presently affiliated with American Breeders Service, Madison, Wis.; Dr. Campbell, Laboratory of Neurological Research, College of Medical Evangelists, Los Angeles County Hospital, Los Angeles, Calif.

The study was supported by a grant from the National Research Council, Committee on Sex, and by funds provided by the Agricultural Experiment Station. The authors are indebted to Miss Margie Slints and to Mr. Shrae LaPlante for technical assistance.

Materials and Methods

The preputial muscles and their nerve supply were dissected in detail in 3 animals (18-month-old bull, 6-weeks-old bull calf, and 1-year-old ram). The number and diameter of the myelinated fibers in the lateral thoracic and inguinal nerves were determined from photographs of microscopic sections of the nerves. Details of the method used have been described previously.[6]

For the electrophysiologic experiments, essentially the same equipment was used as has been previously described.[9] In general, it consisted of condenser-coupled differential amplifiers, a dual-beam oscilloscope, and continuous-recording oscilloscope camera, and a Grass S4 stimulator. Silver wire electrodes were used to stimulate and record responses of nerves submerged in warm (38 to 40 C.) mineral oil. For electromyographic recording, bipolar steel electrodes were inserted into the protractor and retractor preputial muscles. Eighteen animals were used in this series of experiments. Twelve of these were rams about 1 year old. The remainder were bull calves, 1 to 6 months of age. All animals were anesthetized, using allobarbital with urethane* administered intravenously to effect. Gross anatomic observations were made immediately after euthanasia at the conclusion of each experiment.

Observations and Results

The protractor preputial muscles originate adjacent to each other from the external surface of the abdominal cutaneous muscle and subcutaneous tissue in the region of the xiphoid cartilage. They diverge caudally to run approximately 1½ inches from each other at the umbilicus. The muscles unite at a midline raphe, caudal to the preputial orifice. The retractor preputial muscles arise bilaterally from the dartos and abdominal tunicae, caudal to the spermatic cord.** Fibers of each mus-

* Ciba Pharmaceutical Products, Inc., Summit, N.J., supplied the aqueous solution, containing 10 per cent allobarbital (Dial) with 40 per cent urethane.

** The retractor preputial muscles have been reported to be absent in some specimens.[16]

cle extend cranially, mostly lateral to the spermatic cord. They intermingle with those of the opposite side to insert on the prepuce just caudal to the insertion of the protractor preputial muscles.

Branches from the lateral thoracic nerve go to the protractor and retractor preputial muscles. [9] This nerve originates from the ventral branches of C8, Th1, and Th2.

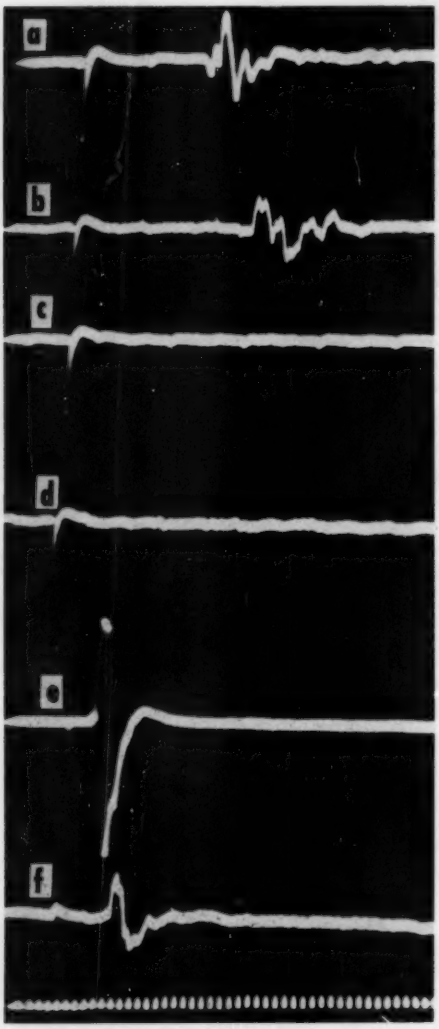


Fig. 1—Electromyographic responses in the protractor preputial muscle (a,c,e) and the retractor preputial muscle (b,d,f) of a calf following stimulation of the caudal inguinal nerve (a,b), caudal inguinal nerve with the lateral cervical nerve transected (c,d), and the distal segment of the transected lateral thoracic nerve (e,f). Time is in milliseconds.

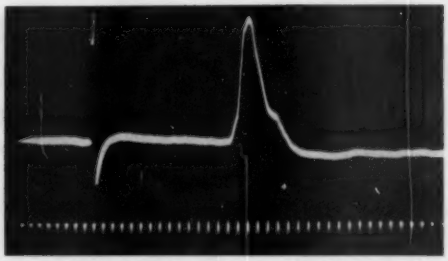


Fig. 2—Reflexly elicited action potential recorded from the lateral thoracic nerve of a ram. The stimulus was delivered to the caudal inguinal nerve. The action potential is bimodal and the earlier component is predominant. Time is in milliseconds.

Single shock stimuli were delivered to the central cut ends of various cutaneous nerves in the anesthetized animals in an effort to find one or several nerves which would consistently cause a reflex contraction of either the protractor or retractor preputial muscle, or both (preputial twitch reflex). The caudal inguinal nerve was the most effective in this regard. In all animals, relatively weak stimulation of the central end of the transected nerve resulted in an ipsilateral, regular contraction of both muscles (fig. 1). Stimulation of the central ends of transected cutaneous branches of the ventral branches of the upper lumbar and lower thoracic nerves also caused contraction of both muscles, but this was usually accompanied by a strong contraction of the abdominal cutaneous muscles and the abdominal muscles. In some animals, strong stimulation of the superficial perineal branch of the pudendal nerve, the dorsal nerve of the penis, or the pudendal nerve resulted in an ipsilateral contraction of the preputial muscles.

Transection of the lateral thoracic nerve abolished the preputial twitch reflex (fig. 1). This indicated that the efferent limb of this reflex was conveyed by this nerve.

Since this reflex is somewhat unique, considering the distance the impulses must travel, it seemed appropriate to further analyze the total time consumed during the reflex. This interval consisted of the time needed to traverse the afferent limb, the central relay, and the efferent limb. In both rams and bull calves, the total time of the reflex from the site of stimulation of

the caudal inguinal nerve to the recording site in the protractor muscle was approximately 14 msec.; in the retractor muscle, it was approximately 19 msec. (fig. 1). The initial deflections of the electromyographic records were variable and difficult to interpret. Using 5 rams, a series of 68 recordings were made of the time consumed from the time of stimulation of the caudal inguinal nerve to the appearance of the action potential at the cut end of the lateral thoracic nerve near the protractor muscle. This interval varied between 14 and 16 msec. (fig. 2).

To calculate time consumed in the afferent and efferent limbs of this reflex, one must know the speed of conduction of the nerve impulse and the distance the stimulating or recording electrodes are from the spinal cord. Additional information can also be secured from histograms of the diameters of the myelinated fibers in these nerves.

Only a single recording of the conduction of impulses in the caudal inguinal nerve was made in a bull calf (fig. 3). The conduction rate in the caudal inguinal nerve in this instance was 60 meters per second.

The diameters of the myelinated fibers in the caudal inguinal nerve were determined in 2 bull calves (fig. 3). This nerve has a fiber diameter spectrum profile with a large number of fibers in the 4- to 8-μ groups, with relatively few fibers having diameters greater than 12 μ. At the point where these samples were taken, which was the site of stimulation in the experiments, the histograms were similar to those usually described for cutaneous nerves. However, as reported earlier,[9] the caudal inguinal nerve also supplies the cremaster muscle. Thus, the nerve has a motor component which leaves the nerve proximal to the point at which these sections were made.

The conduction velocity of the fibers in inguinal nerves can be calculated by using the findings of Hursh,[5] who found that, when the diameter of the fiber is multiplied by six, the conduction time is closely approximated. Using this method of calculation, the largest numbers of fibers in the caudal inguinal nerve would have conduction velocities of 24 to 60 meters per second. The latter corresponds favorably with the determined maximum velocity. Using a conduction velocity of 60 meters per second and an afferent distance of 15 to 18 cm., a

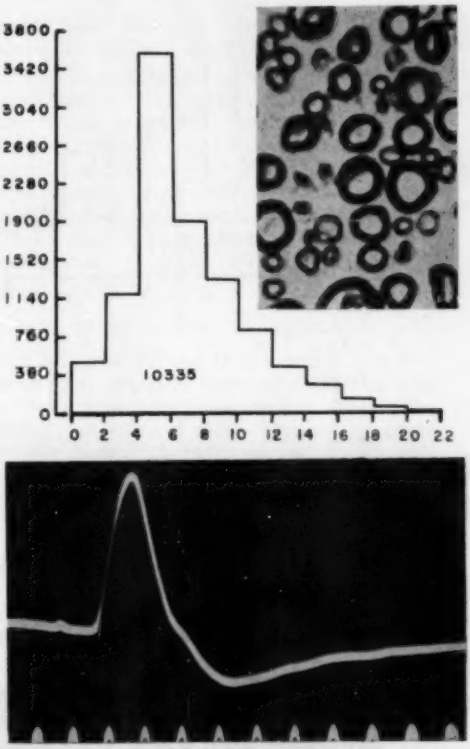


Fig. 3—At the upper right is a photomicrograph of a representative area of a microscopic section of the caudal inguinal nerve of a calf; a modification of Wolters' staining technique was used (x 750). The histogram illustrates the distribution of the diameter of the fibers in the nerve. Horizontal scal diameters (μ); vertical scale, numbers of fibers. Below: the compound action potential recorded from the dorsal root of L4 following stimulation of the caudal inguinal nerve. Time is in milliseconds.

delay of 3 msec. can be accounted for in traversing the caudal inguinal nerve in the afferent limb of this reflex.

The conduction velocity for impulses in the lateral thoracic nerve was found to be approximately 75 meters per second in the bull calf (fig. 4). In most recordings of the reflex discharge from the lateral thoracic nerve, the recording electrodes were placed 17 to 19 cm. from the emergence of the lateral thoracic nerve from the spinal cord. This would indicate that approximately 2.5 msec. would thus be utilized in the efferent limb of the reflex.

The diameters of the myelinated fibers

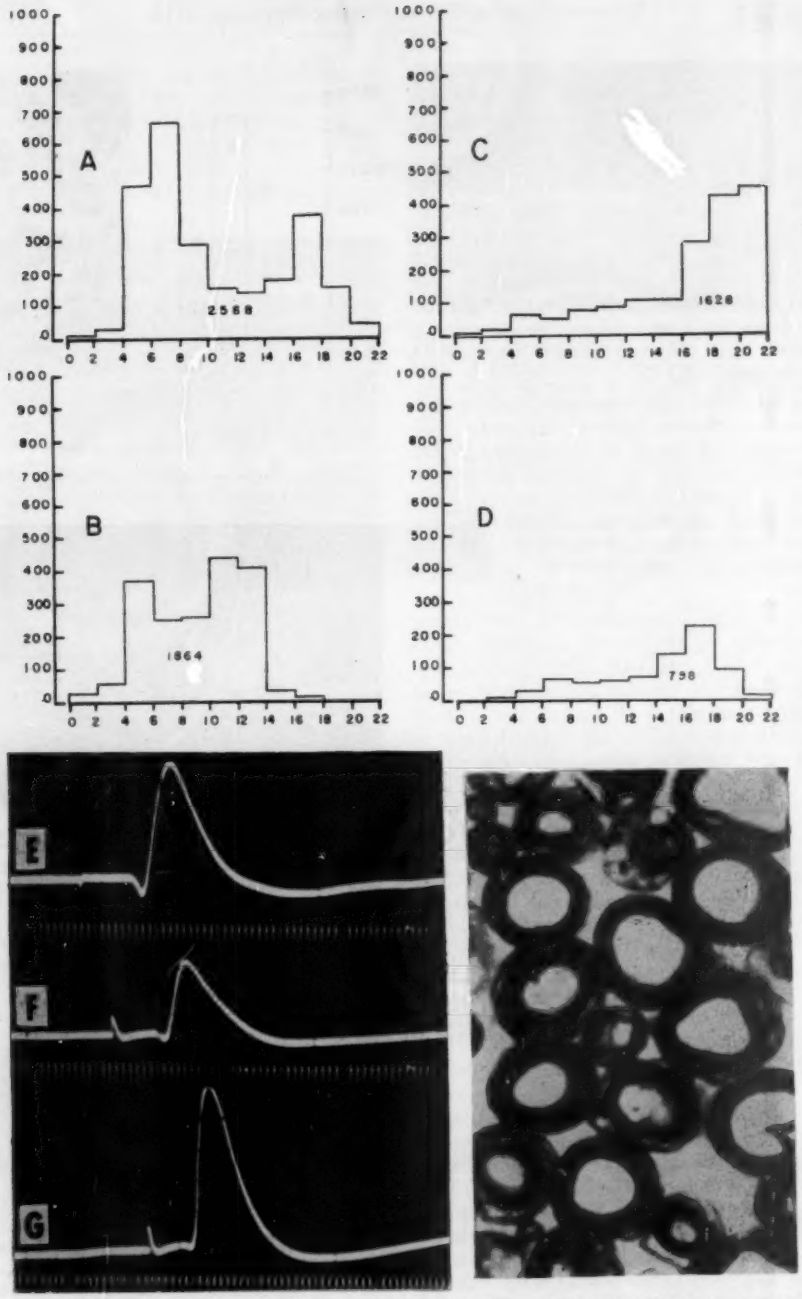


Fig. 4—Histograms showing distribution of fiber diameters in the lateral thoracic nerves of 3 rams (A,B,C) and a young bull calf (D). Horizontal axis indicates the fiber diameter (μ); vertical axis, number of fibers. Lower right: photomicrograph from a typical area of a section of the lateral thoracic nerve from which histogram C was obtained; a modification of Wolters' staining technique was used (x 750). Lower left: compound action potential recorded from the lateral thoracic nerve of a ram following stimulation of the nerve 13 cm. proximally (E), 11 cm. proximally (F), and 9 cm. proximally (G). Time is in milliseconds.

in the lateral thoracic nerve were determined in 3 rams and 2 bull calves (fig. 4). In each animal, the majority of fibers fell in two fiber-diameter groups. The smaller fibers were 4 to 8 μ in diameter; the larger fibers were 10 to 14 μ in calves and 14–20 μ in rams. The number of smaller fibers varied considerably in the animals. During gross dissection, cutaneous branches of the intercostal nerves were seen to join the lateral thoracic nerve. The number of small fibers in each section was related directly to the proximity of the collection site to the point of anastomosis between any cutaneous branch and the lateral thoracic nerve.

Using Hursh's formula with the diameters of the largest fibers in the lateral thoracic nerve of the calf (12 to 14 μ), the calculated conduction velocity of fibers in this nerve is 72 to 84 meters per second. This is in general agreement with the measured velocity of 75 meters per second.

With the afferent and efferent limb conduction times calculated to be 3.0 msec. and 2.5 msec., respectively, the time consumed in the spinal cord would be 9 to 10 msec. The average rate of transmission within the spinal cord can be calculated by using this time and the distance between L3 and Th2 (38 to 42 cm. in the bull calves). Using the mean of these figures, the average rate of central transmission was found to be 4 meters per second.

Discussion

The results of this investigation show that the preputial muscles receive their motor nerve supply by means of branches of the lateral thoracic nerve. This nerve, as well as the sensory nerves to the skin adjacent to the preputial orifice, remains functional following the application of the pudental nerve block. Thus, reflex constriction of the preputial orifice may still occur.

The sensory nerves involved in the preputial twitch reflex were shown to be ventral cutaneous branches of the lower thoracic and lumbar nerves, the inguinal nerves, and the lateral cutaneous femoral nerves. The calculated rate of transmission of the impulses along the spinal cord between these nerves and the lateral thoracic nerve was 4 meters per second. This conduction rate, and the length of spinal cord involved in the reflex, fits the classification of a "long spinal reflex" as proposed by Sherrington and Laslett.[15] The scratch reflex and hand-foot reactions are other examples of long spinal reflexes.

The lateral thoracic nerve has fibers with diameters similar to those of the fibers in the caudal laryngeal and the spinal accessory nerves. All of these nerves are primarily motor nerves. The sensory fibers, which may be present in them, are the result of anastomoses with other nerves. These sensory fibers reach the central nervous system by means of other nerves in all three instances.

Summary and Conclusions

The nerve supply to the protractor and retractor preputial muscles of the bull calf and the ram were investigated. The motor supply was found to be terminal branches of the lateral thoracic nerve. Reflex contraction of these muscles was produced by stimulating the ventral cutaneous branches of the lower thoracic and the lumbar nerves. The inguinal and the lateral cutaneous femoral nerves were particularly effective in eliciting this reflex. As none of these nerves are blocked by the pudendal nerve block, it is proposed that this reflex is responsible for constriction of the preputial orifice during attempts to protract the penis following a pudendal nerve block.

References

[1] Birmingham, A.: Homology and Innervation of the Achselbogen and Pectoralis Quartus, and the Nature of the Lateral Cutaneous Nerve of the Thorax. J. Anat. & Physiol., 23, (1889): 206–223.

[2] Campbell, B.: The Comparative Myology of the Forelimb of the Hippopotamus, Pig and Tapir. Am. J. Anat., 59, (1936): 201–247.

[3] Chaveau, A.: Comparative Anatomy of Domesticated Animals. 2nd ed. D. Appleton & Co., New York, N.Y. (1905): 858, 862, 979.

[4] Ellenberger, W., and Baum, H.: Handbuch der Vergleichenden Anatomie der Haustiere. Bearbeitet von O. Zeitzchmann—E. Ackerknecht—H. Grau. 18th ed. Springer-Verlag, Berlin, (1943): 284, 542–543, 547, 887, 905, 931.

[5] Hursch, J. B.: Conduction Velocity and Diameter of Nerve Fibers. Am. J. Physiol., 127, (1939): 131, 139.

[6] Kitchell, R. L., Campbell, B., Quilliam, T. A., and Larson, L. L.: Neurological Factors in the Sexual Behavior of Domestic Animals. Proc. Book, AVMA, (1955): 177–189.

[7] Langworthy, O. R.: The Panniculus Carnosus in Cat and Dog and Its Genetical Relation to the Pectoral Musculature. J. Mammol., 5, (1924): 49–63.

[8] Larson, L. L.: The Internal Pudendal (Pudic) Nerve Block for Anesthesia of the Penis and Relaxation of the Retractor Penis Muscle. J.A.V.M.A., 123, (July, 1953): 18–27.

[9] Larson, L. L., and Kitchell, R. L.: Neural Mechanisms in Sexual Behavior. II. Gross Neuroanatomical and Correlative Neurophysiological Studies of the External Genitalia of the Bull and the Ram. Am. J. Vet. Res., 19, (Oct., 1958): 853–865.

[10] Michelsson, G.: Die Hautmuskulature des Igels

(Erinaceus Europaeus). Morphol. Jahrb., 51, (1921): 147–227.

[11] Montane, L., and Bourdelle, E.: Anatomie régionale des animaux domestiques. II. Ruminants. Baillière et Fils, Paris (1917): 279, 282, 285, 316, 319, 330.

[12] Nickel, R., Schummer, A., and Seiferle, E.: Lehrbuch des Anatomie des Haustiere. Paul Parey, Berlin, 1954.

[13] Rooney, James R.: Guides to the Dissection of the Horse. 3rd ed. J. W. Edwards, Inc., Ann Arbor, Mich., 1956.

[14] Ruge, G.: Die Hautmuskulature der Monotremen und ihre Beziehungen zu dem Marsupial und Mammarapparate. Aus Semon, Zoolog. Forsschungs-reisen. II. Jena. Denkschr., 5, (1895): 1–153.

[15] Sherrington, C. S., and Laslett, E. E.: Observations on Some Spinal Reflexes and the Interconnection of Spinal Segments. J. Physiol., 29, (1903): 58–96.

[16] Sisson, S., and Grossman, J. D.: The Anatomy of the Domestic Animals. 4th ed. W. B. Saunders Co., Philadelphia and London (1953): 596–601, 836, 862.

[17] Tobler, L.: Der Achselbogen des Menschen, Ein Rudiment des Panniculus Carnosus der Mammalier. Morphol. Jahrb., 30, (1902): 453–507.

SUMMARIO IN INTERLINGUA

Mechanismos Neural in le Comportamento Sexual. III. Le Musculos Preputial e Lor Reflexos in Vitellos Mascule e in Arietes

Le provision de nervos al musculos preputial protractori e retractori esseva investigate in vitellos mascule e in arietes. Esseva trovate que le provision motori es brancas terminal del nervo thoracic lateral. Contraction reflexe del musculos preputial esseva producite per stimular le brancas cutanee ventral del nervos thoracic inferior e lumbar. Le nervos inguinal e femoral cutanee lateral esseva particularmente efficace in le evocation de iste reflexo. Viste que nulle de iste nervos es blocate per bloco de nervo pudental, le these es proponite que iste reflexo es responsabile pro le constriction del orificio preputial durante effortios de protraher le penis post un bloco de nervo pudental.

Experimental Immunization Against Ovine Vibriosis. I. The Use of Live and Formalin-Killed *Vibrio fetus* Vaccines

VICTOR A. MILLER, D.V.M., M.S., and RUE JENSEN, D.V.M., Ph.D.

Fort Collins, Colorado

OVINE VIBRIOSIS, an acute contagious disease, is characterized by abortion during late pregnancy and is caused by *Vibrio fetus*. The disease was first described in ewes in England in 1913,[8] and in New York in 1919.[2] *Vibrio fetus* is considered the most frequent cause of bacterial abortion in ewes in the United States,[9] and has been reported in major sheep-producing areas of the world.

The economic importance of the disease may be evaluated by the incidence of abortion, which varies from 20 to 70 per cent in infected flocks,[4, 10] and by the mortality of aborting ewes, approximately 5 per cent.[4]

In sheep, the reservoir of infection has not been determined.[10] Several investigators have induced abortion in susceptible ewes by oral inoculation or by feeding vibriosis-infected fetal lamb tissues, fluids, or cultures of *V. fetus* of ovine origin.[5-8, 16] These reports indicate that infection occurs through the digestive tract in susceptible pregnant ewes. The demonstration of a bacteremin following oral inoculation with *V. fetus* cultures confirms that one portal of entry into the animal's body is the digestive tract.[16]

A survey of literature reveals that little work has been done on artificial immunization against infection with *V. fetus*.

In 63 vibriosis-negative cows and heifers, administration of three subcutaneous injections of formalin-killed bacterin of *V. fetus* was of no value in preventing the spread of vibriosis.[15] Three subcutaneous injections of a vaccine prepared from *V. fetus* after unspecified numbers of passages through chicken embryos resulted in a conception rate of 66 per cent in a group of 9 vaccinated heifers as compared to 30 per cent in 10 unvaccinated controls when their immunity was challenged by intra-uterine inoculation of a freshly isolated strain of *V. fetus*.

Immunity was demonstrated in 44 ewes one year after abortion caused by vibriosis. An additional 44 ewes, fed infected ovine fetal tissues as virgin yearlings in 1955, proved to be immune when given infected ovine fetal tissues orally during the fifth month of gestation in 1956.[4] Recently, administering *V. fetus* bacterin plus adjuvant to ewes prior to and after breeding resulted in significant protection when immunity was challenged with virulent *V. fetus* during advanced stages of gestation.[1]

This study was conducted to determine vibriosis immunity in ewes given either live or formalin-killed *V. fetus* vaccine prior to breeding.

MATERIALS AND METHODS

Ewes.—Two hundred and ten 2-year-old, primigravid ewes were allotted at random to nine isolated lots. The ewes had been obtained from New Mexico as weanlings in the fall of 1957. They were maintained on plains pasture at Ordway, Colo., until late summer of 1958 when they were placed in experimental pens at the Animal Disease Station, Colorado State University. The ewes of each lot were bred by normal, healthy rams obtained from flocks having no history of vibriosis.

Prior to breeding, ewes of lots 1, 4, and 7 were given two separate 5-ml. subcutaneous injections of viable *V. fetus* culture; ewes of lots 2, 5, and 8 were given two separate 5-ml. subcutaneous injections of formalin-killed culture (table 1).

Ewes of lots 3, 6, 7, 8, and 9 were used as controls for various phases of the experiment.

Ewes in lots 3 and 6 were not vaccinated and served for determining the efficacy of the method of challenge.

Ewes in lot 7, vaccinated with live culture but without subsequent challenge, served as controls for live *V. fetus* vaccine studies.

Ewes of lot 8, vaccinated with formalin-killed culture without challenge, served as controls for formalized bacterin studies.

Ewes of lot 9, nonvaccinated and not challenged, served as controls for the entire experiment.

Ewes in lots 1, 2, 3, 7, 8, and 9 were maintained separately in individual pens within their respective lots from the time of oral challenge with virulent *V. fetus* culture. Each ewe was maintained separately until it had either lambed or aborted.

Ewes in lots 4, 5, and 6 mingled freely with ewes which were aborting from experimental vibriosis.

Received for publication Jan. 15, 1960.

From the College of Veterinary Medicine and the Agricultural Experiment Station, Colorado State University, Fort Collins.

Supported in part by the Agriculture Research Service, USDA.

TABLE 1—Vaccination Against Vibriosis and Challenge of Immunity

	Vaccination with *V. fetus*						Challenge with *V. fetus*				
Lot No.	No. of preg. ewes	Type of vaccine	Time of vaccination	Amount of vaccine (ml.)	Route of inoculation	No. of injections	Density of cell suspension (McFarland tube 1X)	Route	Amount of culture (ml.)	Density of cell suspension (McFarland tube No. 5)	No. of infected lambs	% infected lambs
1	22	live	pre-pregnancy	5	Subcut.	2	20	oral	10	5	0	0
2	25	dead	pre-pregnancy	5	Subcut.	2	20	oral	10	5	7	28
3	25	none	.....	0	.....	..	....	oral	10	5	21	84
4	22	live	pre-pregnancy	5	Subcut.	2	20	cohabitation	0	..	0	0
5	23	dead	pre-pregnancy	5	Subcut.	2	20	cohabitation	0	..	1	4
6	21	none	.....	0	.....	..	....	cohabitation	0	..	8	38
7	24	live	pre-pregnancy	5	Subcut.	2	20	none	0	..	0	0
8	23	dead	pre-pregnancy	5	Subcut.	2	20	none	0	..	0	0
9	25	none	.....	0	.....	..	....	none	0	..	0	0

Experimental Vaccines.—Cultures used for vaccines were prepared from *V. fetus* organisms recently isolated from frozen viscera obtained from a lamb aborted by an ewe given an oral inoculation of *V. fetus* culture in the spring of 1958. Original *V. fetus* cultures were isolated from viscera of aborted lambs obtained from Minturn, Colo., and have been used in experimental studies at this station since 1956. Pathogenicity of the *V. fetus* organism has been maintained by oral inoculation of primigravid ewes at the beginning of their fifth month of gestation. *Vibrio fetus* cultures for the preparation of vaccines were grown in cysteine trypticase broth (0.5 Gm. of l-cysteine, 20 Gm. of trypticase, 5 Gm. of sodium chloride, and 0.5 Gm. of sodium sulfite in 1 liter of distilled H_2O; pH adjusted to 7.2) layered over trypticase soy agar (B.B.L.). The cultures were incubated aerobically in horizontal Blake culture bottles for 42 hours at 37 C. Following incubation, the cultures were examined for purity, for catalase activity, and for hydrogen sulfide production. The bacterial growth from a number of bottles was pooled and then diluted with cysteine trypticase broth until the optical density corresponded to 20 times tube 1 of the McFarland nephelometer standards. (The 1 per cent solutions of barium chloride and sulfuric acid used in preparing the standards were made up in cysteine trypticase broth instead of distilled water.)

The average viable cell count of the diluted vaccine was 7.6×10^9 organisms per milliliter at the time of vaccination. Each ewe of lots 1, 4, and 7 was given a single subcutaneous injection of 5 ml. of viable *V. fetus* culture, repeated once at a two-week interval prior to breeding.

A killed *V. fetus* vaccine was prepared by adding 40 per cent formaldehyde to the unused portion of the viable culture to give a final concentration of 0.2 per cent. The culture was then incubated at 37 C. for 48 hours. The sterility of the bacterial vaccine was determined by placing samples of the formaldehyde-treated culture into screw-capped culture tubes of cysteine trypticase broth containing 0.1 per cent agar and incubating the tubes both aerobically and under reduced oxygen tension. The pH of the vaccine was adjusted to 7.4 by the addition of ammonium hydroxide. Each ewe of lots 2, 5, and 8 was given a single subcutaneous injection of 5 ml. of the formalin-killed culture, repeated once at a two-week interval prior to breeding.

Challenge.—At the beginning of the fifth month of gestation, the immunity of each ewe was challenged by one of two methods: (1) oral inoculation with cultures of recently isolated *V. fetus* obtained from aborted lambs, and (2) cohabitation with aborting ewes that had been experimentally infected with *V. fetus*.

Twenty-five ewes, bred earlier than ewes of other lots, were orally inoculated with recently isolated, viable *V. fetus* cultures. The source of these cultures was described earlier under the

preparation of vaccines. These ewes were used to provide a source of natural exposure for ewes of lots 4, 5, and 6 and to provide a source of fresh culture of *V. fetus* for oral challenge of the immunity of ewes in lots 1, 2, and 3.

Pooled broth cultures of *V. fetus* used for oral challenge of the immunity of vaccinated ewes of lots 1, 2, and 3 were diluted with cysteine trypticase broth until the density, adjusted photoelectrically, corresponded to the McFarland cysteine trypticase broth tube 5. Each ewe was given 10 ml. of viable *V. fetus* whole broth culture orally. The average viable cell count of the challenge inoculum was 2.20×10^9 organisms per milliliter immediately following challenge.

Diagnosis.—Aborted lambs, stillborn lambs, or lambs dying several days following birth were necropsied, and the causative factor was diagnosed as vibriosis by use of one or all of the following criteria: (1) culture isolation and identification of the *V. fetus* organism; (2) presence of *V. fetus* as determined from stained smears made from chorionic cotyledons, uterine exudate, and fetal stomach contents; and (3) presence of characteristic liver lesions.

Results

In the ewes of lots 1, 2, and 3, challenge by oral inoculation with virulent *V. fetus* cultures during the fifth month of gestation resulted in no abortions in ewes of lot 1, while 7 ewes of lot 2 aborted. Ewes of lot 3, nonimmunized and serving as controls for virulence of *V. fetus* culture used in oral challenge, had 21 abortions.

TABLE 2—Chi-Square Analysis for Evaluating the Difference in Numbers of Abortions for Lots

Comparison between lots	P
1 vs. 2	<0.01
2 vs. 3	<0.01
4 vs. 5	<0.5
5 vs. 6	<0.01
2 vs. 5	<0.05
3 vs. 6	<0.01

Immunity of the ewes of lots 4, 5, and 6 was challenged, during their fifth month of gestation, by cohabitation with ewes aborting from oral inoculation with *V. fetus* culture. No abortions occurred in ewes of lot 4, but one abortion occurred in a ewe of lot 5. Eight abortions occurred in lot 6 ewes which were nonimmunized and served as control animals for efficacy of challenge by cohabitation with aborting ewes.

No abortion occurred in ewes of lots 7 and 8 which served as control animals for those given live and formalin-killed *V. fetus*. No abortion occurred in ewes of lot 9 which served as control animals for the entire experiment.

Chi-square probabilities for the data are given (table 2). According to the analysis of the data, protection against infection with *V. fetus* was obtained by the use of vaccines prepared from live and formalin-killed cultures.

Discussion

The general consensus is that ewes aborting from natural vibriosis are immune for at least one year following abortion.

In 1957-1958, vaccinal immunity was demonstrated in a lot of 20 primigravid ewes when their immunity was challenged orally with virulent *V. fetus* culture at the beginning of their fifth month of gestation. Prior to breeding, these ewes were given two subcutaneous injections of viable cultures of *V. fetus*.

A replicate experiment conducted in 1958-1959 again demonstrated that vaccinal immunity against vibriosis may be induced in sheep. Ewes of lot 1, immunized with live *V. fetus*, withstood oral challenge with virulent *V. fetus* administered during the fifth month of gestation. These findings confirm the results of an experiment performed in 1957-1958, in which 20 ewes were similarly immunized.

Formalin-killed *V. fetus* was not as efficient an immunizing agent as live *V. fetus*. This was demonstrated in ewes of lots 2 and 5. The inability of killed suspensions of bacteria to induce as solid an immunity as live cultures is generally admitted.

Summary

To determine immunogenicity of live and formalin-killed cultures of *Vibrio fetus*, immunizing agents were administered in two parenteral doses prior to the breeding of 210 2-year-old ewes, allotted to 9 lots. Challenge of immunity was made at the beginning of the last month of gestation and was by two methods: (1) oral inoculation with viable cultures of *V. fetus* and (2) cohabitation with ewes which were affected with and aborting from vibriosis.

No abortions occurred in ewes given viable *V. fetus* as the immunizing agent. Of the ewes vaccinated with killed *V. fetus*, 28 per cent aborted when immunity

was challenged by oral inoculation. Of the ewes vaccinated with killed *V. fetus*, 4 per cent aborted when their immunity was challenged by cohabitation. Of the nonvaccinated ewes in control lots, 84 per cent aborted when their immunity was challenged by oral inoculation; 38 per cent aborted when challenge was by cohabitation.

References

[1] Boyes, B. W.; Wallaceville Animal Research Station, Wellington, New Zealand: Personal communication, 1959.

[2] Carpenter, C. M.: Researches Upon a Spirillum Associated with Abortion in Ewes. Report, New York State Veterinary College, Cornell (1918–1919): 129–157.

[3] Frank, F. W., Bailey, J. W., and Heithecker, D.: Experimental Oral Transmission of Vibrionic Abortion of Sheep. J.A.V.M.A., 131, (Nov. 15, 1957): 472–473.

[4] Jensen, R., Miller, V. A., Hammarlund, M. A., and Graham, W. R.: Vibrionic Abortion in Sheep. I. Transmission and Immunity. Am. J. Vet. Res., 18, (April, 1957): 326–329.

[5] Lee, A. M., and Scrivner, L. H.: An Investigation of Severe Losses from Abortion in Ewes. J.A.V.M.A., 92, (Jan., 1938): 68–72.

[6] Lee, A. M., and Scrivner, L. H.: Experimental Work Upon Recent Outbreaks of Abortion in Ewes. Am. J. Vet. Res., 2, (Jan., 1941): 50–54.

[7] Lindenstruth, R. W., Ashcroft, J. B., and Ward, B. Q.: Studies on Vibrionic Abortion of Sheep. J. A. V. M.A., 114, (April, 1949): 204–205.

[8] McFadyean, J., and Stockman, S.: Abortion in Sheep. In "Report of the Departmental Committee Appointed to Inquire Into Epizootic Abortion." Part 3. Great Britain Board of Agriculture and Fisheries. His Majesty's Stationery Office, London, 1913.

[9] Marsh, H.: Vibriosis of Sheep. Yearbook of Agriculture, 1956, USDA, 425–426.

[10] Marsh, H.: Newsom's Sheep Diseases. 2nd ed. Williams & Wilkins Co., Baltimore, (1958): 406.

[11] Miller, V. A., Hammarlund, M. A., and Jensen, R.: New Aspects of Vibriosis in Sheep. Proc. III Symp. Reprod. & Infert., Pergamon Press, (1958): 15–19.

[12] Miller, V. A., and Jensen, R.: Unpublished data, College of Veterinary Medicine and Agricultural Experiment Station, Colorado State University, Fort Collins, Colo., 1960.

[13] Miller, V. A., Jensen, R., and Gilroy, J. J.: Bacteremia in Pregnant Sheep Given *Vibrio fetus*. Am. J. Vet. Res., 20, (July, 1959): 677–679.

[14] Osborne, J. C.: Avianized *Vibrio fetus* Vaccine and Some Preliminary Observations on Its Use. Proc. Book, AVMA (1952): 112–115.

[15] Plastridge, W. N., Williams, L. F., Chernak, C., and Easterbrooks, H. L.: The Effect of Vibriosis on Breeding Efficiency in Cattle. J.A.V.M.A., 118, (June, 1951): 367–373.

[16] Tucker, J. O., and Robertstad, G. W.: Experimental Vibriosis in Sheep. J.A.V.M.A., 129, (Dec. 1, 1956): 511–513.

SUMMARIO IN INTERLINGUA

Immunisation Experimental Contra Vibriosis Ovin. I. Le Uso de Vaccinos de *Vibrio fetus* Vive e Morte per Formalina

Pro determinar le immunogenicitate de culturas de *Vibrio fetus* vive e morte per formalina, agentes immunisatori esseva administrate in duo doses parenteral ante le copulation de 210 oves feminin de 2 annos de etate, distribuite in 9 gruppos. Le provocation del immunitate esseva effectuate al commenciamento del secunde mense del gestation. Duo methodos esseva usate: (1) Le inoculation oral de viabile culturas de *V. fetus* e (2) le cohabitation con oves afficite de vibriosis e suffrente abortos como effecto de illo.

Nulle abortos occurreva in oves tractate con viabile *V. fetus* como agente immunisatori. Inter le oves vaccinate con morte *V. fetus*, 28 pro cento suffreva abortos quando le immunitate esseva provocate per inoculation oral. Inter le oves vaccinate con morte *V. fetus*, 4 pro cento suffreva abortos quando le immunitate esseva provocate per cohabitation. Inter le nonvaccinate oves del gruppos de controlo, 84 pro cento suffreva abortos quando le immunitate esseva provocate per inoculation oral e 38 pro cento quando le provocation se faceva per cohabitation.

Pingue (Hymenoxys richardsonii) Poisoning in Sheep

W. A. AANES, D.V.M., M.S.

Fort Collins, Colorado

PINGUE *(Hymenoxys richardsonii,* syn. *Actinea richardsonii),* also known as Colorado rubber weed, is widely distributed from southwestern Canada to Texas. The plant has caused extensive sheep losses in areas of Colorado, New Mexico, and Arizona. Poisoning resulting from ingestion of this plant occurs in areas where pingue is abundant and when the sheep are extremely hungry.

Detailed descriptions of the signs and pathology resulting from poisoning by this weed are not recorded. The purpose of this study was to determine the signs and pathology resulting from acute pingue poisoning in sheep.

REVIEW OF LITERATURE

The first published description of poisoning due to the ingestion of pingue appears to be that of Glover.[5] He described extensive sheep losses in the Middle Park area of Colorado in the month of July and reported that the plant contains no poison but forms an indigestible, rubbery mass in the stomach, which proves fatal by obstructing the bowels. Baker[1] also reported that the ingestion of pingue by sheep caused the formation of rubber-like balls in the rumen and a rubber-like obstruction of the intestinal tract.

In 1929, Marsh[7] stated that experimental work had proved that the plant was poisonous to sheep, but it had been found to be difficult to make them eat it under corral conditions. Field observations have shown that poisoning occurred under conditions approaching starvation.

Parker[6] reported that the greatest loss occurred during the spring and fall seasons and that a positive correlation was found between overgrazing and death losses due to pingue.

The signs of pingue poisoning described by Pammel[5] were bloating, frothing at the mouth, marked cerebral disturbances, convulsions, and recumbency. Baker[1] stated that affected animals became drowsy, lay down, and refused to rise. Death occurred after a course of one hour to ten days.[5, 10]

In 1931, Clawson[4] reported that a small annual weed, known as bitterweed, had become an increasingly serious menace as a sheep poisoning plant. This plant, *Actinea odorata,* is related to the Colorado rubber weed *(A. richardsonii),* and the two plants produce similar effects in sheep.

Experimental work on bitterweed poisoning in sheep by Hardy *et al.*[9] showed that feeding by grazing was unsatisfactory for the production of signs and that force-feeding resulted in signs that varied according to the amount of bitterweed consumed. Signs observed in acute bitterweed poisoning were complete anorexia, cessation of rumination, depression, abdominal pain as evidenced by an arched back and slow movements, bloat, light froth at the mouth, and a dark green discharge from the nose. The pulse rate became accelerated and irregular. Respiration was not accelerated, but labored breathing, accompanied by audible râles, was recorded. The temperature was normal to subnormal. Vomition occurred in 1 of 17 animals. The most common postmortem finding was pulmonary congestion which "approximated hepatization." Hemorrhages were frequently seen on the epicardium and less commonly in the costal pleura and submaxillary and retropharyngeal lymph nodes. There were a few congested areas of gelatinous edema on the surface of the rumen. Other pathologic changes included congestion and hemorrhage on the abomasum, intestines, and, especially, the duodenum. The spleen was tumescent and the parenchyma was slightly softer than normal. The kidneys were congested and friable.

Clawson[4] found that the toxicity of the bitterweed may be cumulative. Daily consumption of bitterweed, amounting to as little as 0.1 per cent of the sheep's weight, induced illness after 44 days. Larger daily doses caused sickness in a correspondingly shorter time.

Boughton and Hardy[3] demonstrated that the minimum lethal dose of bitterweed for a healthy sheep is approximately 1.3 per cent of the body weight when fed as a single dose. They also found that in a drought year (1934), the minimum lethal dose dropped to as low as 0.5 per cent of the animal's body weight and that the state of nutrition had little, if any, effect on the animal's susceptibility to bitterweed poisoning.

In 1956, Boughton[2] reported that the toxic principle or principles of *H. richardsonii* and *A. odorata* are as yet unknown.

Received for publication Jan. 27, 1960.

From the Department of Veterinary Clinics and Surgery, College of Veterinary Medicine, Colorado State University, Fort Collins. Approved for publication as Paper No. S.S. 646 by the reading committee.

Taken from a thesis submitted by the author to the Graduate School, Colorado State University, in partial fulfillment of the requirements for the M.S. degree.

The author thanks Dr. Rue Jensen for the photograph used in this report.

MATERIALS AND METHODS

Plants.—When pingue was in blossom, approximately 75 lb. of the plant was collected by hand near Fairplay, Colo. The stems and foliage were comminuted by passing them twice through a Bolinder Ideal No. 308 grinder with 12-mm. orifices in the grinder plate. The comminuted plant was then quick-frozen and stored at −18 F. until used.

Green alfalfa was collected and processed in the same manner as the pingue. The ground green alfalfa was fed to the control animals.

Animals.—Thirty yearling range sheep of both sexes were used in this project. The males were castrated. Sheep were fed alfalfa hay free choice prior to the experiment.

Twenty-four sheep were force-fed ground pingue. Six sheep were force-fed alfalfa and served as controls (table 1).

TABLE 1—Experimental Animals and Treatments

Lot No.	No. of sheep	Treatment
1	14	Lethal dose of pingue
2	10	Sublethal dose of pingue
3	6	Alfalfa

Prior to experimental feeding, the right carotid artery was cannulated and the blood pressure measured, using a U-tube mercury manometer. A second blood pressure measurement was made on each sheep 24 hours after feeding.

After the first blood pressure recording, the animals were taken off feed for 24 hours. Temperature, pulse, and respiration were recorded. The sheep were then weighed, and the amount of pingue or alfalfa to be fed was determined. The quantity of pingue varied from 1.0 to 3.25 per cent of the animal's body weight. The amount of alfalfa fed was equal to 2.5 per cent of the animal's body weight.

Blood Analyses.—Blood samples were collected by venipuncture of the jugular vein, using a 16-gauge, 2-inch needle, and 3 cc. of blood was placed in a tube containing heparin as an anticoagulant. Blood smears were made directly from the needle, air dried, and stained with Wright's stain. Differential white blood cell counts and packed cell volumes were obtained using standard techniques.

Urine Analyses.—Urine samples were obtained after the collection of blood samples by creating a state of partial asphyxia. Partial asphyxia was established by grasping the muzzle so that the animal was unable to breathe through the nostrils or mouth. Urine tests were conducted with standard techniques. Blood and urine samples were collected at 24-hour intervals following feeding until the animals either died or recovered.

Feeding Procedure.—The animals were restrained in stanchions during the feeding process. The specified amount of plant material was mixed with sufficient water to form a suspension that would pass through a Koroseal* stomach tube with an inside diameter of 0.5 inch. One end of the stomach tube was attached to a wine press and the opposite end was passed down the esophagus into the rumen. With aid of an assistant, the feed material was placed in the wine press and forced by positive pressure through the tube into the rumen of the animal. Any plant material remaining in the press was mixed with more water and forced into the sheep's stomach before the tube was withdrawn.

* B. F. Goodrich Co., Akron, Ohio.

TABLE 2—Vital Statistics for Experimental Sheep

Lamb No.	Sex	Weight (lb.)	Amount of plant (% of body weight)	Time of death post-feeding
LOT 1				
506	F	46	3.25	44 hours
501	F	55	3.0	27 hours
509	M	61	3.0	24 hours
510	M	75	3.0	24 hours
511	F	64	3.0	36 hours
512	M	75	3.0	36 hours
513	M	71	3.0	44 hours
514	M	59	3.0	44 hours
505	M	64	2.67	5 days
515	F	67	2.0	29 hours
516	M	60	2.0	36 hours
518	M	63	1.5	36 hours
519	F	66	1.5	36 hours
523		died from pneumonia		
LOT 2				
503	M	57	2.85	13 days
504	F	62	2.76	13 days
502	M	67	2.5	15 days
507	F	45	1.5	6 days
517	F	73	1.5	14 days
520	F	74	1.5	14 days
508*	F	57	1.0	6 days
521*	F	66	1.0	8 days
522*	F	64	1.0	12 days
524*	M	74	1.0	6 days
LOT 3				
525*	M	87	2.5	21 days
526*	M	82	2.5	30 days
527*	M	64	2.5	30 days
528*	M	80	2.5	19 days
529*	M	82	2.5	19 days
530*	F	88	2.5	19 days

Lot 1 and 2 sheep were fed pingue, and lot 3 sheep were controls fed alfalfa. Lot 1 sheep died, and lot 3 sheep recovered; sheep in lots 2 and 3 were killed.

* No signs of illness.

Following feeding, the temperature, pulse, and respiratory rates, along with other clinical signs, were observed and recorded at varying intervals. These observations began at the time of feeding and were continued until death or recovery of the animals. Recovery was considered complete when appetite, behavior, and bowel movements were again normal.

Pathologic Examination.—Necropsy was performed as soon as practical following death of the animals fatally poisoned with pingue. Sheep that recovered from pingue poisoning and the control animals were killed for postmortem study at the intervals indicated (table 2).

At necropsy, gross tissue changes were recorded and tissue samples were collected from the heart,

lungs, liver, kidneys, spleen, thymus, postpharyngeal lymph nodes, mandibular lymph nodes, abomasum, small intestine, large intestine, bladder, adrenals, and brain. These tissues were fixed in 10 per cent buffered formalin. Histologic sections were prepared from the collected tissues, using a standard technique. Sections were prepared from the cerebrum, thalamus, midbrain, cerebellum, and medulla oblongata. The brain sections were stained with Nocht's stain. All other sections were stained with hematoxylin and eosin. The slides were examined with a microscope and the results recorded.

The differences between prefeeding and 24-hour postfeeding values were obtained for blood pressure, temperature, total white blood cell count, differential white blood cell count, and packed cell volume. Data obtained from sheep that were fed a lethal dose of pingue (lot 1) were compared with data from those given a sublethal dose of pingue (lot 2) and with data from the controls (lot 3). The data were analyzed, using the unpaired Student's "t" test. The *chi*-square test was used to analyze data on the specific gravity, reaction, and occult blood tests of urine samples.

RESULTS

Fourteen of 24 sheep died after being fed varying amounts of pingue (table 2). Six sheep had clinical signs of illness and recovered. Four of the 24 sheep had no signs of illness. One sheep fed pingue died of acute pneumonia; its data are not included in the analysis. The control sheep had no clinical evidence of illness.

Clinical Signs.—Salivation was observed two to three hours after feeding and varied from slight to marked. Depression was seen in most animals approximately four hours after ingestion of pingue. Rumen movements appeared to cease when the sheep became depressed. As depression increased, swallowing movements, grinding of the teeth, and groaning were observed. A slight to moderate bloat developed in some sheep.

Fig. 1—Pingue (Hymenoxys richardsonii)

Affected sheep had a tendency to lie down

TABLE 3—Unpaired Student's "t" Test, Comparing Data from Animals Given a Lethal Dose of Pingue (Lot 1), a Sublethal Dose of Pingue (Lot 2), and Controls (Lot 3)

	Blood pressure (mm. Hg)	Temperature (F.)	Total w.b.c.	Differential count (%)					Packed cell volume (%)
				Seg.	Band	Eosino.	Lympho.	Mono.	
LOT 1VS2									
s	9.90	0.4330	18.00	7.88	0.88	1.93	7.84	0.88	2.42
$\bar{d}$									
$\bar{x}_1 - \bar{x}_2$	−1.56	0.44	26.2	24.00**	0.3	0.5	−22.5*	1.0	4.7
df	19.0	19.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0
LOT 1VS3									
s	7.47	0.401	17.18	7.63	0.70	2.02	6.66	0.89	1.86
$\bar{d}$									
$\bar{x}_1 - \bar{x}_3$	−7.36	1.84**	23.3	25.6**	2.3**	0.17	−26.8**	0.67	6.3**
df	15.0	15.0	14.0	14.0	14.0	14.0	14.0	14.0	14.0
LOT 2VS3									
s	11.98	0.5836	22.54	7.56	0.63	2.28	7.57	1.19	1.91
$\bar{d}$									
$\bar{x}_2 - \bar{x}_3$	−5.80	1.40*	−2.9	1.6	2.0*	0.33	−4.3	0.33	1.6
df	14.0	14.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0

* Significant at the 0.05 level. ** Significant at the 0.01 level.

after becoming ill. They appeared to have difficulty in assuming a comfortable position and moved frequently. When forced to a standing position, weakness was evidenced by incoordinated movements. After lying down, some sheep held their heads in lateral recumbency. A watery diarrhea developed in 8 sheep 12 to 24 hours following the feeding of pingue. Enophthalmos developed rapidly in these animals.

When depression became pronounced, shallow, erratic respirations were observed. In the lungs, congestion and an increase in moist râles was evident upon auscultation.

TABLE 4—Data Obtained from Urinalysis Before and 24 Hours After Feeding

Animal No.	Specific gravity		Reaction (pH)		Occult Blood	
	0	24 hr.	0	24 hr.	0	24 hr.
LOT 1						
501	.....	.....	....	....	.....	.....
505	.....	.....	....	....	.....	.....
506	.....	.....	....	....	.....	.....
509	1.031	1.001	7.5	7.5	Pos.*	Pos.
510	1.045	1.003	7.5	5.0	Pos.	Pos.
511	1.045	1.001	7.5	7.0	Neg.**	Pos.
512	1.040	1.008	7.5	7.5	Neg.	Pos.
513	QNS†	1.005	7.5	7.5	Neg.	Pos.
514	1.045	1.007	7.5	7.5	Neg.	Pos.
515	1.035	1.010	7.5	7.5	Neg.	Pos.
516	QNS	1.012	7.5	6.0	Neg.	Pos.
518	1.030	QNS	7.5	4.5	Pos.	Pos.
519	1.002	1.008	7.5	5.0	Pos.	Pos.
LOT 2						
502	.....	.....	....	....	.....	.....
503	.....	.....	....	....	.....	.....
504	.....	.....	....	....	.....	.....
507	.....	.....	....	....	.....	.....
508	.....	.....	....	....	.....	.....
517	1.028	1.005	7.5	7.5	Pos.	Pos.
520	1.040	1.013	5.0	7.5	Pos.	Pos.
521	1.043	1.045	7.5	7.5	Neg.	Neg.
522	QNS	1.020	7.0	7.5	Neg.	Neg.
524	QNS	1.030	7.5	5.0	Neg.	Neg.
LOT 3						
525	QNS	QNS	7.5	7.5	Neg.	Pos.
526	1.020	1.025	7.5	7.5	Neg.	Pos.
527	QNS	QNS	7.5	7.5	Pos.	Pos.
528	QNS	QNS	7.5	5.0	Neg.	Neg.
529	QNS	QNS	7.5	7.5	Pos.	Pos.
530	1.040	1.041	7.5	7.5	Neg.	Neg.

* Positive for occult blood. ** Negative for occult blood. † Quantity of urine not sufficient to measure specific gravity.

A mucous discharge from the nose was seen in some sheep. Pulse rates were erratic, and no specific pattern was observed.

Significant changes did not occur in blood pressure measurements taken before and 24 hours after feeding pingue (table 3).

The body temperature of sheep that died from pingue was significantly higher ($p<0.01$). The maximum temperature recorded in the first 24 hours following feeding was 105.7 F., while the minimum temperature was 100.6 F. The increase in temperature was greater in the sheep that died than in those fed a sublethal dose (table 3).

Death occurred in one to five days in sheep given a lethal amount of pingue (table 2).

The single minimum lethal quantity in weight of pingue was equal to 1.5 per cent of the sheep's body weight (table 2). Sheep fed pingue equal to 1.0 per cent of their body weight had no signs of illness. All sheep fed pingue in an amount equal to 1.5 per cent or more of their body weight had signs of illness. Animals fed a lethal dose did not eat. Sheep with clinical signs after a sublethal dose had anorexia lasting one to six days. As the lethal quantity was approached, the period of anorexia was extended.

TABLE 5—Gross Pathologic Findings in Numbers of Sheep Affected (Lot 1)

Location	No. sheep affected
CONGESTION	
Mandibular lymph nodes	6
Retropharyngeal lymph nodes	8
Rumen	4
Abomasum	12
Lungs	12
Trachea	3
HEMORRHAGE	
Epicardium	10
Endocardium	9
Rumen	3
Abomasum	12
Small intestine	3
Pleura	6
EDEMA	
Rumen	11
Lungs	8
Thoracic cavity	3
Peritoneal cavity	9
Bronchi	9

Blood Analyses.—Applying the unpaired Student's "t" test, a significant increase in segmented cells occurred when sheep in lot 1 were compared to sheep in lots 2 and 3 (table 3). There was a significant increase in band cells in lot 2 sheep as compared to lot 3 sheep ($p = <0.05$). A more significant increase in band cells occurred in sheep fed a lethal dose of pingue than in those fed a sublethal dose. A significant drop in number of lymphocytes occurred in lot 1 sheep as compared to those of lot 3 (table 3). The packed cell volumes of

sheep fed a lethal dose of pingue increased significantly ($p<0.01$).

Urine Analyses.—No change was seen in the color or transparency of the urine. *Chi*-square statistical analysis (table 4) indicated a significant reduction in the specific gravity of the urine of sheep fed pingue ($p<0.05$). The occurrence of occult blood in the urine of the controls could not be explained.

In 8 of 24 sheep fed pingue, there was an increase in albumin varying from a trace to a marked amount.

Significant changes were not observed in other urine tests.

Pathologic Examination.—Gross pathologic changes were observed only in sheep given a lethal dose of pingue. The most pronounced lesions occurred in the digestive and respiratory systems (table 5). Histopathologic sectioning of the rumen tissue showed varying amounts of submucosal edema and necrosis of villi with infiltration of polymorphonuclear leukocytes. Occasional hemorrhages occurred in the mucosa of the abomasum. Fatty degeneration of hepatic cells was seen in all zones of the liver lobules.

Discussion

In general, the clinical signs observed in this experiment were similar to those recorded by other authors [3, 4, 6, 8] for pingue and bitterweed poisoning. These signs indicated that pingue causes severe disturbances in the digestive and respiratory systems.

Cerebral disturbances and convulsions described by Pammel [8] were not observed. Sheep given a lethal quantity of pingue did become comatose prior to death. Death occurred one to five days following feeding. Others [8, 10] observed that under natural conditions death occurred from one hour to ten days following ingestion of the plant.

There was no evidence of vomiting or a green discoloration of the nasal discharge as was described by Hardy *et al.*[6]; however, grinding of the teeth, groaning, and swallowing movements were observed and indicated nausea and abdominal pain.

A statistically significant temperature rise was observed in sheep given a lethal dose of pingue in contrast to the report of Hardy *et al.*,[6] in which temperature was normal to subnormal in sheep poisoned with bitterweed.

There was no evidence of the formation of rubber-like balls of feed material in the digestive tract as reported by Glover [5] and Baker.[1] If sublethal amounts of the weed had been fed over an extended period of time, this condition might have developed.

The pathologic changes were similar to those described for bitterweed poisoning.[6] It is highly probable that the two plants contain the same toxic principle[s]. The gross and microscopic pathologic changes indicate that the toxin has a direct action on the digestive system and is absorbed, causing degenerative changes in the liver.

Summary

Twenty-four sheep were fed pingue. Six sheep were fed alfalfa and served as controls. Acute poisoning was produced, causing death in one to five days. The clinical signs and the pathologic changes indicated the occurrence of severe disturbances in the digestive and respiratory systems. The minimum lethal dose of pingue was 1.5 per cent of the sheep's body weight.

References

[1] Baker, E. T.: Sheep Diseases. 2nd ed. Alexander Eger, Chicago, 1929.

[2] Boughton, I. B.: College Station, Texas: Personal communication, 1956.

[3] Boughton, I. B., and Hardy, W. T.: Toxicity of Bitterweed (*Actinea odorata*) for Sheep. Texas Agric. Exper. Sta. Bull. 552, (1937): 1–15.

[4] Clawson, A. B.: A Preliminary Report on the Poisonous Effects of Bitter-Rubber-Weed (*Actinea odorata*) on Sheep. J. Agric. Res., 43, (1931): 693–701.

[5] Glover, G. H.: Report of the Veterinarian. Colorado Agric. Exper. Sta. Rep., (1905): 51–61.

[6] Hardy, W. T., Cory, V. L., Schmidt, H., and Dameron, W. H.: Bitterweed Poisoning in Sheep. Texas Agric. Exper. Sta. Bull. 433, (1931): 1–18.

[7] Marsh, C. D.: Stock Poisoning Plants of the Range. Rev. ed. USDA Bull. 1245, (1929): 1–75.

[8] Pammel, L. H.: Pingue Poisoning in Arizona. J.A.-V.M.A., 14, (1919): 32.

[9] Parker, K. W.: Prevention of Death Losses in Sheep on Areas Infested with Pingue. New Mexico Agric. Exper. Sta. Bull. 241, (1936): 1–74.

[10] Settele, J. M.: Garo, Colorado: Personal communication, 1956.

Summario in Interlingua

Invenenamento de Oves per Pingue (*Hymenoxys richardsonii*)

Vinti-quatro oves esseva alimentate con pingue (*Hymenoxys richardsonii*). Sex oves esseva alimentate con lucerna e serviva como gruppo de controlo. Pingue resultava in in-

venenamento acute, causante morte in inter un e cinque dies. Le signos clinic e le alterationes pathologic indicava le occurrentia de sever disturbationes in le systemas digestive e respiratori. Le minime dose letal de pingue esseva 1,5 pro cento del peso corporee del ove.

Neuropathology of Ovine and Bovine Toxoplasmosis

ADALBERT KOESTNER, D.V.M., M.Sc., Ph.D., and
CLARENCE R. COLE, D.V.M., M.Sc., Ph.D.

Columbus, Ohio

TOXOPLASMOSIS was not recognized in ruminants until 1942, when Olafson and Monlux [11] reported spontaneous toxoplasmosis in a sheep. They noticed muscular rigidity, fever, and respiratory distress. A diffuse, nonsuppurative encephalitis was present, with the most severe lesions in the cervical and thoracic cord. They consisted of monocytic perivascular infiltration, vacuolization of the white matter, and focal accumulations of microglia and monocytes. Pseudocysts were found.

Wickham and Carne,[14] in 1950, reported similar clinical signs in a sheep in Australia. The clinical diagnosis was "circling disease." Microscopic examination of the brain revealed perivascular cuffings and pseudocysts.

Cole *et al.*,[3] in 1954, reported an epizootic of toxoplasmosis in a flock of sheep where 5 ewes and 3 lambs had died. The clinical signs were referrable to the respiratory and central nervous systems. Toxoplasmosis was confirmed by isolation of *Toxoplasma gondii* and by demonstration of organisms in tissue sections. The disease was also reproduced in young lambs and adult sheep, and the clinical signs and the lesions were described.[2, 4] Intrauterine transmission to lambs was confirmed experimentally. Hartley and Marshall,[8] in 1957, considered toxoplasmosis as the most widespread and probably the most important cause of infective ovine perinatal mortality in New Zealand. Of 16 sheep with field cases, 3 had toxoplasma cysts in the brain, 5 had glial nodules with central caseous necrosis, and 8 had focal, noninflammatory necrosis of the cerebral white matter.

Sanger *et al.*, [12] in 1953, isolated *T. gondii* from cattle for the first time. The cattle came from four widely separated herds in Ohio. Congenital Toxoplasma infection in a newborn calf was confirmed by isolation and demonstration of Toxoplasma from emulsions of organs obtained at the time of birth. *Toxoplasma gondii* was also isolated from the milk of the dam. The clinical picture of bovine toxoplasmosis was not uniform but usually included nervous signs.

Sanger *et al.*[12] described the clinical signs and the gross and microscopic lesions in cattle with experimental toxoplasmosis. Cole *et al.*[4] succeeded in intrauterine transmission of the disease to bovine fetuses.

The incidence of toxoplasma infection in sheep and cattle was shown to be unexpectedly high in serologic surveys. Feldman [6] found 50 per cent of the sheep examined in Kentucky to react to the cytoplasm-modifying test for toxoplasmosis. Of two herds of cattle examined in New York, one had 38 per cent and the other 49 per cent reactors.

Cole *et al.* [3] demonstrated Toxoplasma antibodies (titer of 1:8 or greater) in 43 per cent and 44 per cent of the cattle in two herds where the presence of infection was confirmed by identification of Toxoplasma organisms. Both the prevalence and quantity of antibodies were greater in the infected herds than in serum samples from 67 herds selected at random in the same geographic area. Antibodies were detected (titer of 1:8 or greater) in 23 per cent of 400 cattle representing the 67 herds. Six cattle, previously negative at 1:2, developed titer end points only up to 1:8 at 21 days following inoculation with Toxoplasma. This seems to indicate that cattle, in contrast to dogs and man, have a limited antibody response to Toxoplasma.

This investigation was undertaken to (1) describe and classify the lesions of the central nervous system (CNS) in confirmed toxoplasmosis of sheep and cattle; (2) study the frequency and distribution of cerebral lesions in relationship to species and age; (3) compare the findings with those described in dogs and man; and (4) present differential diagnostic criteria of ovine and bovine toxoplasmosis based on the microscopic lesions of the CNS.

MATERIALS AND METHODS

The brains and spinal cords for this investigation are from the collection of the Department of Veterinary Pathology, Ohio State University, and represent animals with previously reported natural and experimental toxoplasmosis.[2-4, 12, 13] Twenty sheep (experimentally infected) and 17 cattle (13 experimentally infected and 4 with naturally occurring cases) were studied. These animals had been either inoculated with virulent organisms (experimental cases), or *Toxoplasma gondii* had been isolated

Received for publication Jan. 29, 1960.

From the Department of Veterinary Pathology, Ohio State University, Columbus.

Supported in part by Grants E-319 and E-2114 from the U.S. Public Health Service, National Institutes of Health.

from or demonstrated in the organs at necropsy (naturally occurring cases). The experimental animals were inoculated orally, subcutaneously, intravenously, or intraperitoneally. Sheep and cattle with congenital cases of toxoplasmosis were included. Emulsions for the isolation of *T. gondii* were prepared with sterile saline solution by grinding the tissues in a mortar without an abrasive. For primary isolations, white mice were inoculated intraperitoneally or intracerebrally. Antibiotics were added to the inoculum. Subinoculations of tissues from mice surviving 30 days after inoculation were accomplished in a similar manner. The technique of isolation was that described in detail by Farrell *et al.*[5] and Chamberlain *et al.*[1] "Demonstration" indicates the recognition of free, intracellular, and encysted forms of *T. gondii* in tissue sections stained with hematoxylin and eosin.

All brains and spinal cords were fixed and stored in 10 per cent isotonic neutral formalin. Fifteen to 25 sections were taken from each animal at various levels of the CNS, including the spinal cord, medulla oblongata, cerebellum, pons, corpora quadrigemina, thalamus, hypothalamus, basal ganglia, and the cerebral cortex. The sections were embedded in paraffin, cut at 6 μ, and stained with hematoxylin and eosin. The following special techniques were used: Von Kossa's stain for phosphates, Van Gieson's stain for connective tissue, Weil-Weigert's myelin stain, periodic acid-Schiff reaction, toluidin blue for Nissl bodies, Gomori's iron reaction, and alizarin red for calcium.

Fetuses and animals up to 5 years of age were examined.

RESULTS

All clinical and laboratory data, including age incidence and distribution of the lesions in the CNS of sheep and cattle, are summarized and listed (tables 1 to 6).

TABLE 1—Relationship of Age to the Occurrence of Lesions and the Presence of Toxoplasma gondii in the Central Nervous Systems of 20 Experimentally Infected Sheep

Age	Lesions	*T. gondii*	Negative
Congenital	3	2	3
1 week	3	3	..
2–5 years	9	5	2
Total	15	10	5

OVINE TOXOPLASMOSIS

Euthanasia or Death Within Ten Days Following Inoculation.—Five animals were included in this group (table 3). Sheep 1 and 2 died on the fourth postinoculation day (p.i.d.), sheep 3 was killed on the eighth p.i.d., sheep 4 was killed on the ninth p.i.d., and sheep 5 died on the tenth p.i.d. All animals were acutely ill, with fever starting 24 hours after inoculation and reaching 107 F. at the second or third day. The clinical signs included râles, dyspnea, anorexia, diarrhea, depression, weakness, trembling, opisthotonus, head tilt, incoordination, dehydration, and abortion. *Toxoplasma gondii* was successfully isolated from 3 of 5 animals and demonstrated in many organs in all 5.

Microscopic Lesions in the Central Nervous System.—The ewe (sheep 1) which died on the fourth p.i.d. had a moderate but generalized congestion of the CNS. Multiple

TABLE 2—Distribution of Lesions and Toxoplasma gondii in the Central Nervous Systems of 15 Sheep

Location	No. of animals	
	Lesions	*T. gondii*
Spinal cord	4	3
Medulla	9	4
Pons	8	5
Cerebellum	10	4
Midbrain	13	7
Cortex and basal ganglia	11	6
Meninges	12	..

small hemorrhages were seen in sections of the brain stem and cerebellum. Endothelial swelling was seen in arterioles, venules, and precapillaries. The perivascular spaces appeared dilated; traces of an eosinophilic proteinic material indicated early inflammatory edema. Venules occasionally had moderate perivascular accumulation of mainly microglia and proliferated adventitial cells. A focus of necrosis was seen in the fasciculus cuneatus of the spinal cord. The focus measured 0.15 mm. in its largest diameter. It consisted of pyknotic nuclei and unidentifiable masses of basophilic material embedded in a meshwork of microglia and a few astrocytes. An amorphous, eosinophilic material formed the background of this lesion. Numerous oval and crescent-shaped extracellular *T. gondii* were present.

In lamb 2, which also died on the fourth p.i.d., the brain was severely affected. The principal lesions were numerous small foci of necrosis with a median diameter of 90 μ. They occurred in apposition with damaged capillaries and precapillaries. Degeneration of the walls of these vessels ranged from simple endothelial swelling to severe necrosis. In advanced lesions, there was marked perithelial cell proliferation, some-

times occluding the vessels completely. Toxoplasma organisms were seen intravascularly as well as in the vascular walls, perivascular spaces, and brain substance surrounding these vessels. Parenchymal necrosis appeared to mark the trail of the parasites which migrated through the vascular walls and multiplied in the perivascular tissue. The cellular reaction ranged from minimal to moderate. Some lesions were detected only by the minute derangement of the smooth central nervous structure. A few microglia, pyknotic nuclei, and nuclear fragments were usually present

TABLE 3—Experimental Ovine Toxoplasmosis

Sheep No.	History	*T. gondii*			Central nervous system
		Age	Isol.	Histol.	
1	Severe illness; died 4th p.i.d.	2 yr.	neg.	pos.	Early vascular injury, focal necrosis, free forms of *T. gondii*.
2	Severe illness; died 4th p.i.d.	1 wk.	pos.	pos.	Severe vascular damage; focal necrosis, extracellular *T. gondii*, and *cysts*.
3	Inappetence, fever, depression; euthanasia 8th p.i.d.	4 yr.	neg.	pos.	Negative.
4	Severe illness; euthanasia 9th p.i.d.	2 yr.	pos.	pos.	Perivascular cuffings.
5	Severe illness, abortion, and nervous signs; died 10th p.i.d.	2 yr.	pos.	pos.	Glial nodules, *cysts*.
6	No clinical illness; euthanasia 13th p.i.d.	1 wk.	pos.	pos.	Glial nodules, *cysts*.
7	No clinical illness; euthanasia 14th p.i.d.	1 wk.	pos.	pos.	Glial nodules, glial and fibrotic scars, medial calcification, *cysts*.
8	Recovery from slight illness; euthanasia 16th p.i.d.	5 yr.	neg.	pos.	Negative.
9	Severe illness; euthanasia 22nd p.i.d.	2 yr.	pos.	pos.	Glial nodules, *cysts*.
10	Severe illness and abortion; euthanasia 23rd p.i.d.	2 yr.	pos.	pos.	Glial nodules, medial calcification.
11	No clinical illness; euthanasia 37th p.i.d.	4 yr.	neg.	pos.	Vascular fibrosis, glial scars, *cysts*.
12	Acute illness, abortion, and gradual recovery; euthanasia 75th p.i.d.	2 yr.	neg.	pos.	Vascular and perivascular gliosis and fibrosis, medial calcification.
13	Acute illness, gradual recovery; euthanasia 111th p.i.d.	2 yr.	neg.	pos. (brain)	Vascular fibrosis and medial calcification, glial nodules, *cysts*.
14	Quick recovery from slight illness; euthanasia 699 p.i.d.	3½ yr.	neg.	neg.	Lesions, nonspecific.
15	Fetus from ewe 3. Cesarean section 2nd p.i.d.; congenital.	unborn	neg.	neg.	Negative.
16	Stillborn from ewe 5 5th p.i.d.; congenital *T. gondii*.	.....	neg.	neg.	Negative.
17	Stillborn from ewe 13 6th p.i.d.; congenital.	.....	neg.	neg.	Negative.
18	Lamb from ewe 4, born 4th p.i.d.; congenital.	1 day	pos.	pos.	Edema, early vascular changes.
19	Born 21st p.i.d. from ewe 8. Euthanasia 6 days postpartum; congenital.	6 days	neg.	pos.	Focal necrosis, glial nodules, free *T. gondii*, and *cysts*.
20	Fetus from ewe 11. Cesarean section 37th p.i.d.; congenital *T. gondii*.	unborn	neg.	pos.	Necrosis, demyelination, glial nodules, *cysts*.

p.i.d. = postinoculation day; isol. = isolation of organisms; histol. = histologic demonstration.

(fig. 1). The cellular reaction in larger foci was more pronounced, with microglial, monocytic, and perithelial cell proliferation. Organisms were present in almost every lesion. They were seen most frequently as extracellular proliferating forms. At the periphery of larger foci, Toxoplasma organisms were intracytoplasmic and occasionally formed small cysts containing 4 to 7 organisms. There was also a moderate infiltration of the meninges by lymphocytes and plasma cells.

While the lesions of the CNS in sheep 4 were very mild and restricted to vessels, they were quite prominent in ewe 5 which died on the tenth p.i.d.

Sequelae of vascular damage were perivascular edema, perivascular as well as diffuse vacuolization (status spongiosus), and perivascular gliosis. The most striking lesion, however, was the scattered appearance of well-defined glial nodules. Their location was always in areas where degeneration of vascular walls could easily be detected, but it was rarely possible to trace the vessel from which the emigration of organisms originated. Thus, the glial nodules were impressive as characteristic and independent lesions. They consisted mainly of pleomorphic and rod cell types of microglia. In addition, there were a few lymphocytes, monocytes, and proliferated perithelial cells. The latter were greatly swollen and degenerated, sometimes karyolytic with

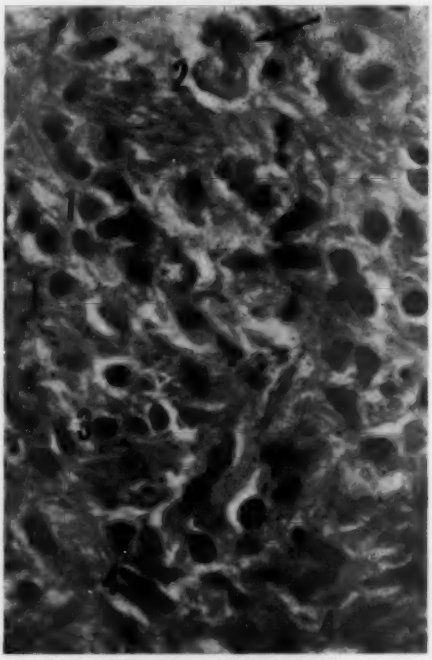


Fig. 2—Early glial nodule in the midbrain of ewe 5, which died on the tenth postinoculation day. Notice pleomorphic microglia (1), vesiculated and karyolytic nuclei of astrocytes (2), activated oligodendroglia (3), elongated irregular perithelial cells (4), and Toxoplasma (arrow). H & E stain; x 1350.

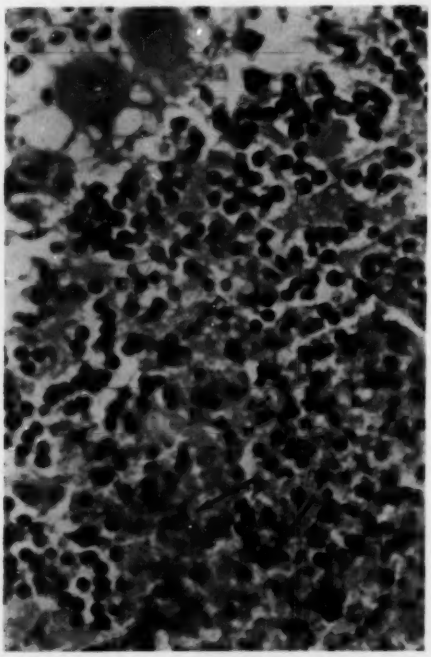


Fig. 1—Pyknosis and karyorhexis (arrows) indicating a focus of necrosis in the granular layer of the cerebellum of lamb 2, which died on the fourth postinoculation day. H & E stain; x 620.

TABLE 4—Relationship of Age to the Occurrence of Lesions and the Presence of Toxoplasma gondii in the Central Nervous Systems of 4 Naturally Infected and 13 Experimentally Infected Cattle

	Naturally occurring			Experimental		
Age	Lesions	*T. gondii*	Neg.	Lesions	*T. gondii*	Neg.
Cong.	..	..	1	2	..	1
0–1 mo.	..	..	1	2	1	3
1–3 mo.	..	..	..	..	..	..
3–6 mo.	..	..	..	1	1	..
6–12 mo.	..	..	..	2	..	..
Over 1 yr.	..	..	2	1	..	1
Total	..	..	4	8	2	5

dumbbell- and handle-shaped nuclei. Regression in the oligodendroglial cells was evidenced by karyorrhexis and karyolysis. The astrocytes were activated, but the swollen, potato-shaped, highly vesiculated nuclei indicated severe degenerative changes with nuclear disintegration (fig. 2). Extracellular *T. gondii* could not be detected, but cysts with large numbers of organisms were present in abundance. As many as 18 cysts were seen in and at the margin of a single glial nodule in a section from the quadrigeminal plate. Ganglion cells, as well as ectodermal glia outside the lesions, also had degenerative changes of varying degrees.

Euthanasia After the Tenth Postinoculation Day.—Nine animals were included in this group (table 3). Some of the severely ill animals which aborted were depressed and dehydrated when euthanasia was performed (sheep 10). Some were killed in a stage of advanced emaciation (sheep 9). Others recovered gradually from severe illness following stillbirth (sheep 13) or abortion (sheep 12). Others recovered quickly from slight fever and coughing (8 and 14), and 3 had no clinical signs of toxoplasmosis following inoculation (6, 7, and 11). In 8 of the 9 sheep, *T. gondii* was demonstrated in organs other than the brain; isolation was successful in 4 sheep. All sheep were destroyed, so it is not known how many might have died as the result of toxoplasmosis.

Sheep 6, 7, and 8 were destroyed on the thirteenth, fourteenth, and fifteenth p.i.d., respectively. There were no lesions in the CNS in sheep 8, but marked and extensive lesions occurred in sheep 6 and 7. The vessels had perithelial cell proliferation with peripheral microglial overgrowth. Prominent cuffings were occasionally seen. The glial nodules were numerous and well developed. They measured up to 0.5 cm. in diameter and were closely packed with cells. Eighty per cent of the cells in the nodule were microglia, two thirds of which were of

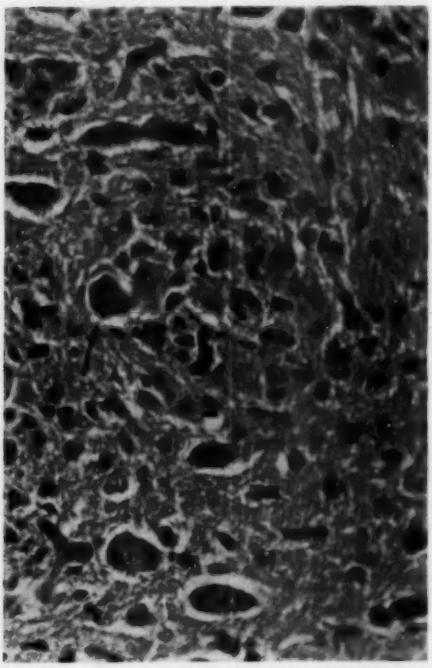


Fig. 3—Well-developed glial nodule in the midbrain of lamb 7 on the fourteenth postinoculation day. Arrow points to a Toxoplasma cyst. H & E stain; x 620.

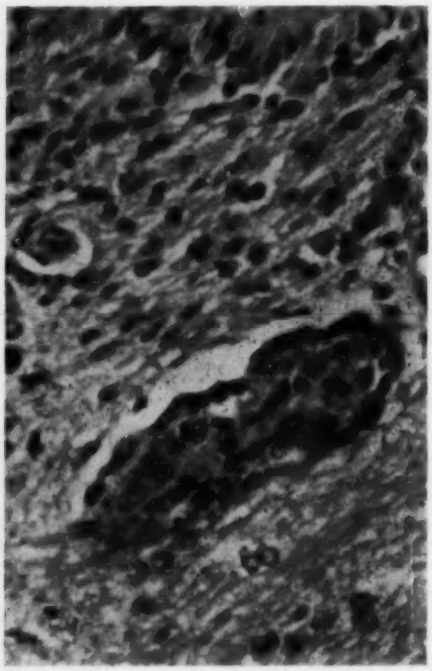


Fig. 4—A glial nodule (top of picture) adjacent to a venule in lamb 7. The wall of the vessel is infiltrated with cells. H & E stain; x 620.

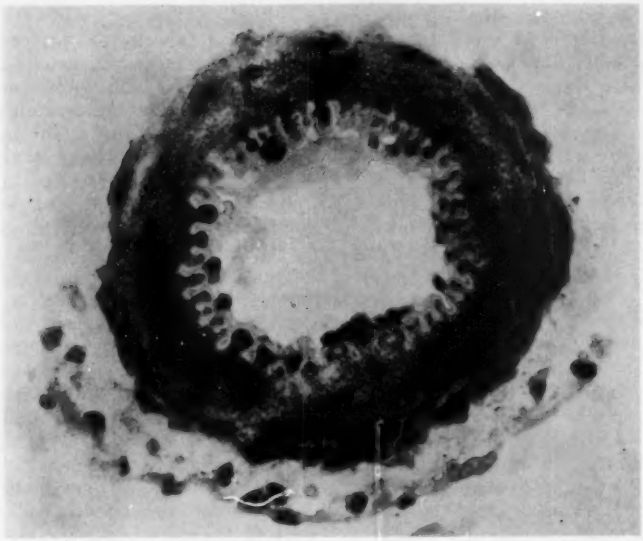


Fig. 5—Marked mineralization of a meningeal vessel in ewe 10. Notice the numerous mineral granules throughout the media and limited mainly by the elastic membrane. Von Kossa's stain; x 690.

the pleomorphic type (fig. 3, 4). Nodules in apposition with arterioles had marked fibrosis characterized by elongated fibroblastic nuclei and formation of fibrillar strands. Toxoplasma cysts within and at the periphery of the lesion were numerous. The meninges in sheep 6 had only slight lymphocytic and plasma cell infiltration.

In addition to the meningitis, the meningeal vessels in sheep 7 had a dense, dark, purplish-blue stained media which was interpreted as early mineralization. Advanced mineralization of meningeal and cerebral vessels was seen in sheep 10, 12, and 13. The material stained positive for phosphates with Von Kossa's technique and was either seen in fine granules or as a solid substance that was dark purplish-blue in sections stained with hematoxylin and eosin (fig. 5, 6). Gomori's stain for iron salts was negative, as was alizarin red for calcium. The positive Von Kossa reaction for phosphates indicated that the material was probably deposited as $CaPO_4$; the calcium was undoubtedly removed during the storage in formalin.

Many vessels in these sheep had prominent cuffings or fibrosis. The glial nodules were densely packed with cells, and the previous defect appeared well repaired. *Toxoplasma gondii* was seen only in cysts. The number of cysts apparently decreased

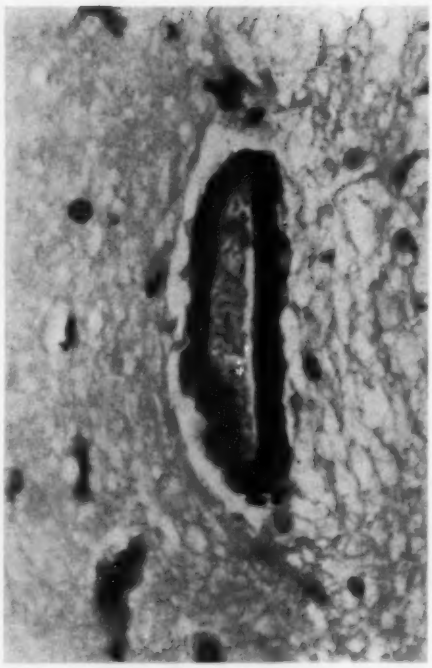


Fig. 6—Complete mineralization of the wall of an arteriole in the cerebral cortex of ewe 10. H & E stain; x 760.

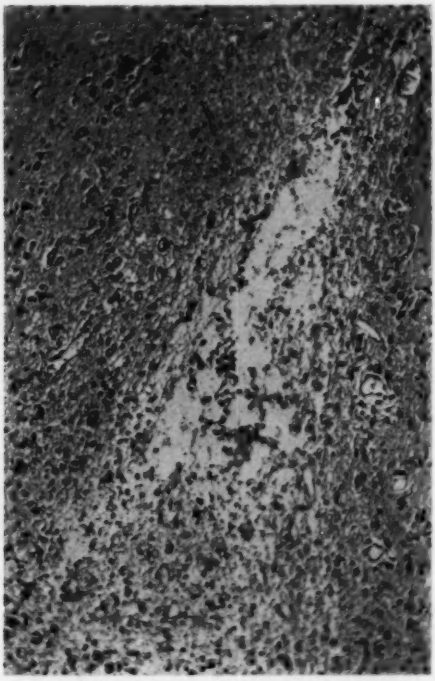


Fig. 7—Extensive demyelination and necrosis in lamb 20. H & E stain; x 125.

with the duration of illness, but an occasional cyst was still observed in sheep 13 on the 111th p.i.d. In this animal, plasma cells were frequently seen perivascularly, and the presence of glial fibrotic scars indicated successful organization of parenchymal defects.

Congenital Toxoplasmosis.—Six sheep with the possibility of congenital toxoplasmosis were examined. All were dead fetuses or live or stillborn lambs from ewes infected during pregnancy. Three of these (15, 16, 17), born on the second, fifth, and sixth day following infection of the dam, were negative on isolation and demonstration of *T. gondii*. Organisms or characteristic lesions in the CNS were not demonstrable.

Case 18.—Euthanasia was performed a few hours postpartum. Parturition (ewe 4) occurred on the fourth p.i.d. *Toxoplasma gondii* was isolated from and demonstrated in many organs of the lamb.

The lesions of the CNS were slight and nonspecific. Endothelial and perithelial swelling and perivascular edema indicated increased vascular permeability. There was generalized congestion and noticeable early cellular infiltration of the leptomeninx.

Case 19.—Euthanasia was performed on the sixth day postpartum. It was the lamb of the ewe 8 which had been infected 21 days prior to delivery. Isolation of Toxoplasma was unsuccessful, but organisms were demonstrated in several organs.

Lesions in various stages of development were seen, ranging from focal necrosis with free organisms to well-defined glial nodules and cysts. A moderate, diffuse meningitis was present.

Case 20.—This lamb was recovered by cesarean section. The lamb was immediately destroyed to determine whether intrauterine infection had occurred. The dam (sheep 8), infected with *T. gondii* 37 days previously, had shown no clinical illness. Organisms were demonstrated in several organs of the lamb, including the placenta, but isolation attempts were negative.

The lesions in the CNS were widespread

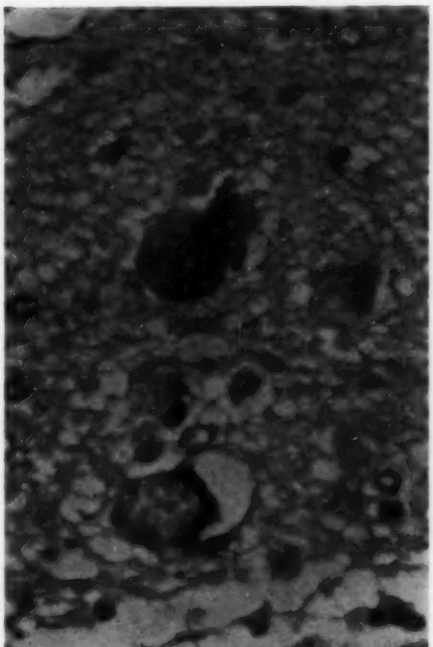


Fig. 8—Higher magnification of tissue section shown figure 7 reveals a cyst within the area of demyelination. Notice swollen endothelial cells of the nearby pericapillary. PAS; x 800.

and numerous in every section of the brain and spinal cord. There was evidence of destruction and repair in almost all vessels. Some vessels were denuded of endothelium and their walls and the Virchow-Robin spaces were heavily infiltrated by perithelial cells, lymphocytes, microglia, and monocytes. Glial nodules were numerous and generally well developed. Others were in an initial stage where necrosis was the prominent feature. Many cysts were found; some were remote from the lesions and were unassociated with any reaction. Numerous organisms were seen in the cytoplasm of macrophages. The nuclei of the phagocytic cells were pushed to the periphery of the cells. A large lesion observed in the pons measured 2.0 by 0.5 mm. and consisted of a central area of necrosis and demyelination with several gitter cells scattered throughout. Changes in the adjacent vessels were as previously described. Diffuse gliosis, glial nodules, and several Toxoplasma cysts surrounded this focus. The cysts were well demonstrated with the PAS reaction; the wall and the central bodies stained strongly positive. They were prominent, well-organized, red spheres that were easily recognized at a magnification of 100 diameters (fig. 7, 8, 9). The meninges were infiltrated by lymphocytes, plasma cells, and monocytes. The ganglion cells within and in the neighborhood of glial nodules and adjacent to severely damaged vessels had mainly ischemic cell necrosis. Various stages of neuronal degeneration were found throughout the brain.

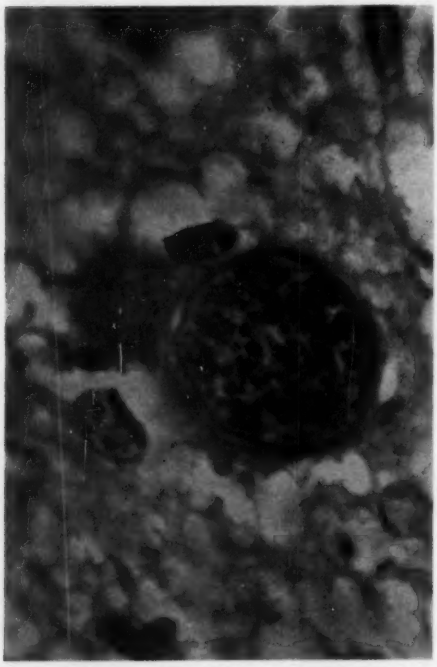


Fig. 9—Tissue section shown in figures 7 and 8; the cyst has the characteristic PAS-positive wall and crescent-shaped organisms with PAS-positive central bodies. PAS; x 1800.

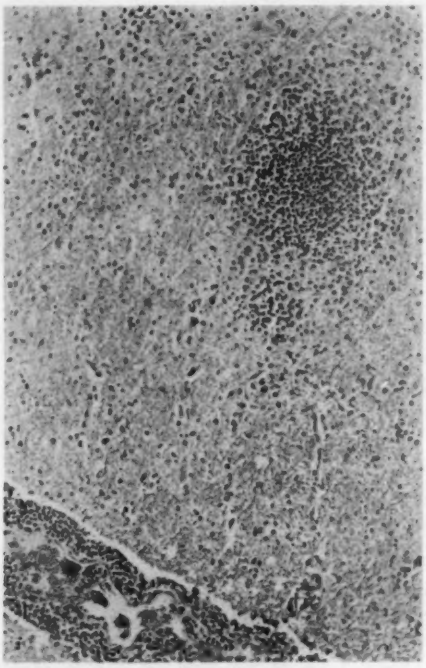


Fig. 10—Microabscess in the medulla of a sheep which died from listeriosis. Notice perivascular cellular infiltration of an adjacent vessel. H & E stain; x 125.

Differential Diagnosis.—Toxoplasmosis in sheep was characterized by high fever, respiratory distress, CNS disturbance, abortion, and premature birth. Lesions in the CNS were among the most constant and characteristic findings in ovine toxoplasmosis. The lesions must be differentiated from those caused by agents other than *T. gondii*. A brief discussion and differen-

tial diagnosis of the most important diseases of the nervous system in sheep seems to be appropriate. Several of them, like listeriosis, have a similar clinical picture and may cause abortion. This discussion will consider primarily the histopathologic differential features of the CNS.

Listeriosis.—Lesions are generally confined to the brain stem, medulla, and spinal cord. The perivascular cuffings are prominent and consist mainly of lymphocytes and monocytes. There is limited tissue necrosis, but microabscesses (fig. 10) may occur and are then diagnostic for listeriosis. Gram-positive, rod-shaped *Listeria monocytogenes* can sometimes be isolated and demonstrated in the CNS. Meningitis usually occurs and is much more severe and extensive than in any case of ovine toxoplasmosis studied. The meningitis is commonly detected clinically on the basis of hyperthermia, hypersensitivity, and hypertonia, and the tentative diagnosis of listeriosis is made.

Scrapie.—In scrapie (Tremblante de Mouton, Traber-Krankheit), a poliomyeloencephalitis, the lesions are predominantly seen in the medulla oblongata, pons, midbrain, and spinal cord. The striking feature is the vacuolization of ganglion cells, which normally occurs to a limited extent in the ovine medulla [15] but is impressively more severe, extensive, and widespread in sheep affected with scrapie.[16] Neuronophagia and slight inflammatory response in the form of perivascular round-cell infiltration may be seen. Lesions rarely occur in the cerebral cortex.

Rabies.—Mainly a polioencephalitis, rabies is characterized by neuronal degeneration ranging from acute swelling to necrosis and neuronophagia. Intracytoplasmic inclusions (Negri bodies) in ganglion cells (in the hippocampus and in Purkinje cells) are pathognomonic. Perivascular lymphocytic infiltration may be slight or extensive and is a common feature of viral diseases of the CNS.

Louping Ill.—The tick *Ixodes ricinus* transmits louping ill, a viral disease found in Scotland, England, and, apparently, in Russia. The gray matter is predominantly affected. The lesions consist of ganglion cell necrosis (especially of Purkinje cells, which explains the ataxia and incoordination), neuronophagia, perivascular cuffings, and focal gliosis.[7]

Swayback.—Involvement of the white matter (leukoencephalomyelitis) is the predominant feature of swayback, which affects young lambs. The lesion consists of demyelination, leaving the axis cylinders intact. The cause of the disease is a copper deficiency or excessive molybdenum in the diet.[9]

None of these five diseases cause lesions which can be easily confused with toxoplasmosis. The principal lesions of the CNS in sheep with acute toxoplasmosis is focal necrosis with numerous free Toxoplasma organisms invading the brain through damaged vessels. The reactive cell is the microglia. In advanced cases, glial nodules are seen which are easily distinguished from microabscesses of listeriosis and from focal gliosis of viral infections. The microabscesses consist of neutrophils and some macrophages. The focal gliosis is a local proliferation of microglia and a response to infection. There is no necrosis related to focal gliosis, and the accumulated cells are exclusively microglia. A glial nodule, as found in toxoplasmosis, has a specific pattern, is generally in the vicinity of damaged vessels, and consists of pleomorphic microglia, rod cells, degenerated astrocytes, oligodendroglia, monocytes, and proliferated perithelial cells. Toxoplasma cysts are often found adjacent to such nodules. The vascular lesions also differ from the perivascular cuffings of viral diseases. They are generally less prominent. The main cell types are microglia and proliferated perithelial cells, not lymphocytes. Fibrosis of vascular walls and glial or fibrotic scars in the parenchyma are seen in sheep with chronic infection.

Another important feature is the distribution of the lesions. In all diseases mentioned, there is a certain restriction to particular areas or there are at least predilection sites known, while lesions due to *T. gondii* are almost equally distributed throughout the CNS. Ganglion cells occasionally undergo degeneration or ischemic necrosis when severe vascular damage prevents proper oxygen supply. On the basis of these criteria, a diagnosis of toxoplasmosis can be made with reasonable accuracy from the lesions alone in the absence of organisms.

Bovine Toxoplasmosis

Euthanasia or Death Within Ten Days Following Inoculation.—Three experimental animals are included in this group.

All 3 became severely ill after infection; 2 died and 1 was destroyed.

Case Reports and Microscopic Lesions in the Central Nervous System.—Calf 1.—This 1½-week-old calf was acutely ill with fever, scours, weakness, depression, and tremor. Euthanasia was performed on the eighth p.i.d. Isolation and demonstration of *T. gondii* were successful.

The lesions in the CNS were essentially restricted to vessels and perivascular regions. Endothelial swelling, thickening of the walls, perivascular edema, and vacuolization of the tissue (fig. 11) were seen. There was minimal lymphocytic infiltration and perithelial cell proliferation. Occasionally, the vascular lesions extended into the adjacent nervous tissue as evidenced by small foci of necrosis, proliferation of microglia, acute swelling of oligodendroglia, and activation of gemistocytes. The foci averaged 200 μ in diameter and did not contain demonstrable organisms.

TABLE 5—Distribution of Lesions and Toxoplasma gondii in the Central Nervous Systems of 8 Cattle

Location	No. of animals	
	Lesions	*T. gondii*
Spinal cord	1	1
Medulla	3	..
Pons	3	1
Cerebellum	7	..
Midbrain	3	1
Cortex and basal ganglia	5	1
Meninges	3	..

Calf 2.—This 4-month-old calf died on the ninth p.i.d. after high fever, anorexia, tachycardia, trembling, and weakness. Isolation and demonstration of organisms were successful.

The vascular lesions in the CNS resembled those of calf 1 but were more severe. The perivascular edema was extensive. There were multiple small hemorrhages. Perivascular cuffings were not observed. Numerous foci of necrosis were seen, especially in the thalamic and cortical sections. They varied from 0.10 to 0.50 mm. in diameter and were in apposition with damaged vessels. Round and oval extracellular *T. gondii* were present in these foci. A few cysts, containing numerous Toxoplasma organisms were seen occasionally at the peripheries of the lesions. The reaction varied in the individual foci from slight to moderate. The microglial cell was predominant. Cells with oblong- or handle-shaped nuclei, originating from the vascular adventitia, were quite numerous. The oligodendroglial cells were present in a normal number, but the nuclear background was lighter and the amount of chromatin was decreased, indicating degeneration. The astrocytes also appeared to be degenerated; their nuclei were enlarged and almost depleted of chromatin. A few monocytes were usually seen and recognized by their round and slightly indented nuclei. There was severe degeneration of ganglion cells in these areas (fig. 12). Moderate round cell infiltration was seen in the meninges.

Fig. 11—Extensive perivascular edema and vacuolization in calf 1. Notice the distended perivascular lymph spaces. H & E stain; x 200.

Calf 3.—This 2-week-old calf died on the tenth p.i.d. following diarrhea and depression. *Toxoplasma gondii* was not isolated but was demonstrated in tissue sections.

Edema, vacuolization, and slight perivascular gliosis of cerebellar vessels was seen in the CNS.

Euthanasia or Death After the Tenth Postinoculation Day.—Seven cattle with experimental cases and 3 naturally infected animals were included in this group.

No lesions of the CNS were observed in 5 of the 10 animals. The remainder had moderate perivascular gliosis, a few glial nodules, and occasional infiltration of the meninges by lymphocytes and plasma cells. In none of the animals could Toxoplasma organisms be demonstrated. Mineralization of meningeal vessels was seen in 2 cattle and a large area of calcification was seen in the medulla of a heifer (fig. 13). The nuclei of the peripheral ganglion cells were also mineralized and the axis cylinders were encrusted or infiltrated, or both, but no glial reaction or vascular damage was seen in serial sections through the entire lesion.

Congenital Toxoplasmosis.—Four calves were studied; 3 were offspring from experimentally infected dams and 1 was a calf from a 5-year-old cow which reacted to the Toxoplasma skin test. *Toxoplasma gondii* was isolated from the milk of this cow. The calf was not permitted to nurse.

The CNS of 1 experimentally infected calf and of the naturally infected calf were negative (14 and 17). The others (15 and 16) had edema, endothelial swelling, perithelial cell proliferation, and perivascular gliosis. A few vessels of the medulla and midbrain of calf 16 were mineralized. Organisms were not identified.

Differential Diagnosis.—Toxoplasmosis apparently is not a common clinical illness in cattle and is not easily reproduced, even though a serologic survey indicated that many cattle are exposed to *T. gondii*.[3] The CNS seems to be especially resistant. From the cases reported here and from experiences with other species, it is concluded that the lesions of toxoplasmosis in cattle are similar to those in dogs and sheep. The differential criteria for other species also apply to toxoplasmosis in cattle.

Toxoplasmosis of the CNS must be

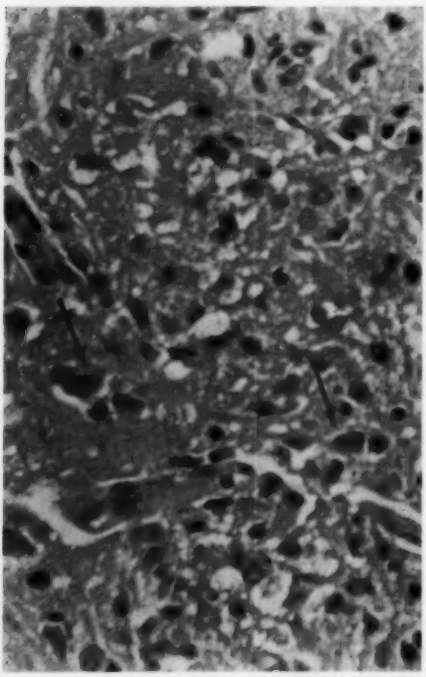


Fig. 12—Early glial nodule with extensive vacuolization in calf 2. Notice the degeneration of ganglion cells (arrows). H & E stain; x 620

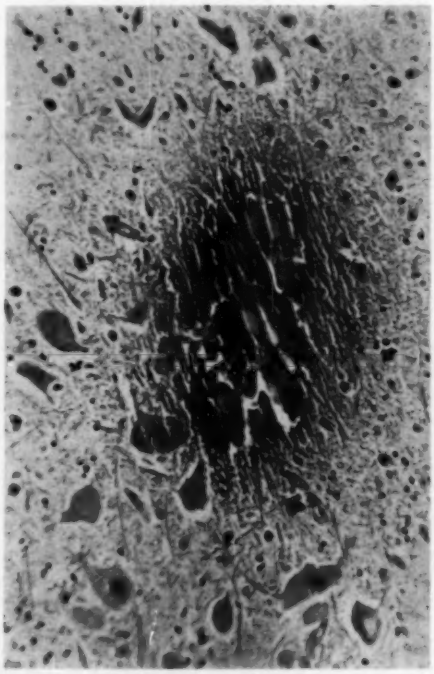


Fig. 13.—Von Kossa-positive plaque of mineralization in the region of the hypoglossal nucleus in cow 8. H & E stain; x 200.

TABLE 6—Experimental and Naturally Occurring Bovine Toxoplasmosis

Animal No.	Mode of infection	History	Age	*T. gondii* Isol.	*T. gondii* Histol.	Central nervous system
1	E	Fever, scours, depression, tremor; euthanasia 8th p.i.d.	1½ wk.	pos.	pos.	Perivascular edema and gliosis, focal gliosis, free *Toxoplasma gondii*.
2	E	Fever, anorexia, weakness, trembling; died 9th p.i.d.	4 mo.	pos.	pos.	Vascular damage, focal necrosis, early glial nodules, free *T. gondii*, and *cysts*.
3	E	Diarrhea, depression; died 10th p.i.d.	2 wk.	neg.	pos.	Edema, vacuolization, perivascular gliosis.
4	E	Fever, depression, recovery; euthanasia 15th p.i.d.	4 wk.	neg.	neg.	Negative.
5	E	Recovery from mild illness; euthanasia 68th p.i.d.	2 yr.	neg.	pos.	Perivascular gliosis, glial nodules.
6	E	Slight fever, recovery; euthanasia 96th p.i.d.	2 yr.	neg.	pos.	Negative.
7	E	No illness; euthanasia 185th p.i.d.	1 yr.	neg.	neg.	Mineralization of meningeal vessels.
8	E	No illness; euthanasia 210th p.i.d.	1½ yr.	neg.	neg.	Perivascular cuffings, cerebral and vascular mineralization.
9	E	No illness; euthanasia 250th p.i.d.	.....	neg.	neg.	Negative.
10	E	No illness; euthanasia 250th p.i.d.	.....	neg.	neg.	Negative.
11	N	Positive skin test; no illness.	5 yr.	pos. (milk)	neg.	No specific lesions.
12	N	Respiratory disease, progressive prostration; killed.	5 wk.	pos.	pos.	Cortical edema.
13	N	Excitement, mashing of jaws, bicycling.	4 yr.	pos. (milk)	neg.	Neuronal degeneration of Purkinje cells, patchy necrosis of the granular layer.
14	E cong.	Healthy calf from cow 5; born 15th p.i.d. Euthanasia.	5 hr.	neg.	pos.	Negative.
15	E cong.	Healthy calf from cow 6; born 60th p.i.d. Euthanasia.	1 hr.	neg.	pos.	Edema, perivascular gliosis.
16	E cong.	Healthy calf from cow 8; born 130th p.i.d. Euthanasia.	1 day	neg.	neg.	Hemorrhage, congestion, perivascular gliosis, area of vacuolization and necrosis.
17	N cong.	Healthy calf from cow 11.	1 day	pos.	neg.	Negative.

Cong. = congenital; E = experimental; N = naturally occurring; isol. = isolation of organisms; histol. = histologic demonstration.

differentiated from rabies, pseudorabies, rinderpest, malignant catarrhal fever, foot-and-mouth disease, sporadic bovine encephalomyelitis, listeriosis, tuberculosis, actinomycosis, trypanosomiasis, and aberrant larvae of metazoa. The first five are virus diseases and are characterized by perivascular lymphocytic cuffings and focal gliosis. The lesions are only occasionally present in rinderpest, malignant catarrhal fever, and foot-and-mouth disease; when present, they occur in both the gray and white matter (panencephalitis). Rabies and pseudorabies are polioencephalitides and, in addition to the cuffings and glial proliferation, result in severe damage of the neurons. Inclusion bodies are found in ganglion cells in both diseases and are considered pathognomonic.

Sporadic bovine encephalomyelitis is a nonsuppurative panencephalitis with numerous elementary bodies demonstrable in the cytoplasm of mononuclear cells.

Listeriosis is probably the most common infection of the CNS in cattle. The lesions, similar to those in sheep and swine, are characterized by meningitis and microabscesses which are valuable diagnostic features in this disease.

Granulomatous inflammation occurs in tuberculosis and actinomycosis. Specific organisms are easily demonstrated with special stains.

Perivascular infiltration of plasma cells and glial nodules are believed character-

istic for infections with *Trypanosoma spp.*[7] Aberrant larvae may lead to necrosis, suppuration, hemorrhage, and demarcation by granulation tissue.

Discussion

The description of the findings in the CNS for ovine and bovine toxoplasmosis clearly demonstrates the similarity of the lesions in these species. In general, the lesions were also similar to those found in dogs.[10] In the early stage, lesions consisted of damage to the vascular walls, as indicated by endothelial swelling, perivascular edema, and proliferation of adventitial cell elements. The conspicuous scarcity of exudative processes, such as diffuse edema, hemorrhage, infiltration of hematogenic cells, and ischemia, was striking. Instead, the process spread to the adjacent nervous tissue, revealing foci of necrosis with numerous extracellular proliferating Toxoplasma organisms. The reaction was slight in the beginning and consisted mainly of microglial proliferation. Glial nodules were found in animals in the subacute and chronic stages. In the latter, glial nodules consisted of pleomorphic microglia, rod cells, activated oligodendroglia and astrocytes, perithelial cells, and monocytes. Toxoplasma organisms were seen intracellularly and in cysts at this stage. Chronic healed toxoplasmosis was characterized by scar formation in all three species. A striking finding in chronic ovine and bovine toxoplasmosis was mineralization of vascular walls. Arterioles and precapillaries were mainly involved; meningeal vessels were more severely affected than cerebral vessels. This lesion was not found in any other species studied and has not been reported previously in the literature. The animals involved (4 sheep and 3 cattle) were 3 weeks to 2 years of age. While arteriosclerosis has been observed in cattle in conditions other than Toxoplasma infection (vitamin D toxicity), it has not been described in sheep. In the microscopic study of brains of sheep which died of diseases other than toxoplasmosis, no calcification of blood vessels was seen.* Therefore, the authors were inclined to attribute this phenomenon to injury by Toxoplasma organisms. In analyzing this substance, it was possible to exclude pseudocalcium, melanin, and iron salts by appearance and special techniques. Using the Von Kossa technique, the material was positive for phosphates, but it did not react to alizarin red for calcium. No doubt the calcium was removed during storage in formalin.

Cerebral calcification, common to chronic toxoplasmosis in children, appears to be rare in animals. One calcified plaque was found in a cow, but its definite relationship to toxoplasmosis could not be demonstrated.

The lesions of the CNS were equally distributed and no predilection site was found. The incidence of cerebral lesions was high in ovine toxoplasmosis (75%) and low in bovine toxoplasmosis (47%). This compares with an incidence of 74 per cent in dogs.[10] This disease was easily reproduced in lambs and in adult sheep up to 2 years of age; lesions in the CNS appeared as early as four days following inoculation. The lesions of the CNS in cattle, however, were mild. Only in 2 animals were Toxoplasma organisms demonstrated in brain and cord sections. Only 1 calf had lesions of an extent comparable to those of other species. This animal had been inoculated intravenously with 30 ml. of infected mouse peritoneal exudate (approximately 360 million organisms). A standard inoculum of 1.6 million organisms was used in our other experiments. While calves were more susceptible than cows, there was no apparent difference in susceptibility between lambs and adult sheep up to 2 years of age (tables 1, 4).

Based on the characteristic findings and the differential diagnostic features, high diagnostic significance is attributed to the lesions of the CNS in ovine and bovine toxoplasmosis.

Summary

The neuropathologic observations on 20 sheep and 17 cattle afflicted with toxoplasmosis are reported. Except for 4 cattle, all animals were experimentally infected with virulent *Toxoplasma gondii*. The 4 cattle had naturally occurring toxoplasmosis, confirmed by isolation or demonstration of organisms. Lesions in the central nervous system (CNS) that were characteristic of toxoplasmosis were found in 15 sheep (75%) and 8 cattle (47%).

Toxoplasmosis of the CNS in sheep and cattle is characterized by focal necrosis and vascular damage in animals with acute infection and by glial nodules, repair, and

* This observation was also confirmed by Dr. Innes who has had extensive experience in ovine neuropathology (personal communication).

vascular mineralization in animals with chronic disease. The differential diagnostic criteria of cerebral toxoplasmosis of sheep and cattle are presented and discussed.

References

[1] Chamberlain, D. M., Docton, F. L., and Cole, C. R.: Toxoplasmosis. II. Intra-Uterine Infection in Dogs, Premature Birth and Presence of Organisms in Milk. Proc. Soc. Exptl. Biol. & Med., 82, (1953): 198–200.

[2] Chamberlain, K. W., Cole, C. R., Koestner, A., and Sanger, V. L.: Toxoplasmosis. VIII. Experimental Toxoplasmosis in Sheep and Lambs. In Preparation, 1960.

[3] Cole, C. R., Sanger, V. L., Farrell, R. L., and Kornder, J. D.: The Present Status of Toxoplasmosis in Veterinary Medicine. North Am. Vet., 35, (1954): 265–270.

[4] Cole, C. R., Chamberlain, K. W., Sanger, V. L., and Koestner, A.: Toxoplasmosis. IX. Experimental Intra-Uterine Transmission of Toxoplasmosis in Cattle and Sheep. In preparation, 1960.

[5] Farrell, R. L., Docton, F. L., Chamberlain, D. M., and Cole, C. R.: Toxoplasmosis. I. Toxoplasma Isolated from Swine. Am. J. Vet. Res., 13, (April, 1952): 181–185.

[6] Feldman, H. A.: Toxoplasmosis; Prevalent in Man and Animals. The Newer Knowledge About Dogs. 6th Gaines Vet. Symp., Kankakee, Ill., 1956.

[7] Frauchinger, E., and Fankhauser, R.: Vergleichende Neuropathologie des Menschen und der Tiere. Springer-Verlag, Berlin, 1957.

[8] Hartley, W. J., and Marshall, S. C.: Toxoplasmosis as a Cause of Ovine Perinatal Mortality. New Zealand Vet. J., 5, (1957): 119–124.

[9] Innes, J. R. M.: Demyelinating Diseases in Animals. A. Swayback: A Demyelinating Disease of Lambs with Affinity to Schilders Encephalitis. B. The Relation of Distemper Infection to the Aetiology of Canine Encephalopathies. In "Multiple Sclerosis and the Demyelinating Diseases." William & Wilkins Co., Baltimore, 1950.

[10] Koestner, A., and Cole, C. R.: Neuropathology of Canine Toxoplasmosis. Am. J. Vet. Res., 21, (Sept., 1960): 831–844.

[11] Olafson, P., and Monlux, W. S.: Toxoplasma Infection in Animals. Cornell Vet., 32, (1942): 176–190.

[12] Sanger, V. L., Chamberlain, D. M., Chamberlain, K. W., Cole, C. R., and Farrell, R. L.: Toxoplasmosis. V. Isolation of Toxoplasma from Cattle. J.A.V..M.A., 123, (Aug., 1953): 87–91.

[13] Sanger, V. L., Chamberlain, K. W., Chamberlain, D. M., and Cole, C. R.: Toxoplasmosis. VII. Experimental Bovine Toxoplasmosis. In preparation.

[14] Wickham, N., and Carne, H. R.: Toxoplasmosis in Domestic Animals in Australia. Austral. Vet. J., 26, (1950): 1–3.

[15] Zlotnik, I., and Rennie, J. C.: The Occurrence of Vacuolated Neurons and Vascular Lesions in the Medulla of Apparently Healthy Sheep. J. Comp. Path. & Therap., 57, (1957): 30–36.

[16] Zlotnik, I.: The Histopathology of the Brain Stem of Sheep Affected with Experimental Scrapie. J. Comp. Path. & Therap., 68, (1958): 428–438.

SUMMARIO IN INTERLINGUA

Neuropathologia de Toxoplasmosis Ovin e Bovin

Es reportate le observationes neuropathologic in 20 oves e 17 pecias de bestial affligite de toxoplasmosis. Con le exception de 4 pecias de bestial, omne le animales esseva inficite experimentalmente con virulente *Toxoplasma gondii*. Le 4 pecias de bestial habeva toxoplasmosis de occurrentia natural, confirmate per le isolation o confirmation del organismo. Lesiones del systema nervose central de typo characteristic de toxoplasmosis esseva trovate in 15 oves (75%) e 8 pecias de bestial (47%).

Toxoplasmosis del systema nervose central in oves e bestial es characterisate per necrosis focal e lesiones vascular in casos acute e per nodulos glial, reparo, e mineralisation vascular in casos chronic. Es presentate e discutite le criterios del diagnose differential de toxoplasmosis cerebral in oves e bestial.

Site of Localization and Effects of Swine Nasal Virus in Experimental Pigs

W. P. SWITZER, D.V.M., Ph.D.; E. D. ROBERTS, D.V.M.; C. L'ECUYER, D.V.M.

Ames, Iowa

UTILIZATION of cell culture techniques has facilitated isolation of viruses from swine nasal cavities. Several properties of 11 cytopathogenic isolates from the nasal cavities of swine in 76 groups have been reported in a previous publication.[5] These isolates produced types of cell destruction which were identified as types 1, 2, and 3.[5]

The present report gives information on the site of localization of these viruses in pigs, their effect upon the host, and information on their serologic relationships.

METHOD OF PROCEDURE

All pigs used in this work were from a disease-controlled swine herd maintained in quarantine and known to be free of detectable respiratory disease since herd establishment over six years ago. Prior to the current work, an enteric virus was isolated from several young pigs in this herd and it was found that some of the pigs 8 weeks old or older had high levels of neutralizing antibodies against this virus. Therefore, preinoculation nasal and rectal swabs from all experimental pigs were processed in primary swine kidney cell cultures for virus detection. Detectable preinoculation viruses were not found in any of the experimental pigs. The procedures used to produce cell cultures and to prepare samples for inoculation have been described previously.[4, 5]

The pigs were removed from the dam 12 to 24 hours after birth and were placed in isolation rooms where they were fed a ration based on cows' milk. Two to ten days after birth, the pigs were inoculated intranasally with 0.5 ml. of cell-culture fluid containing virus. Nasal and rectal swabs were collected four to seven days postinoculation and again ten to 36 days after inoculation. The final samples were collected at necropsy. Tissues were collected and fixed in Bouin's fixative plus 5 per cent acetic acid. Both Giemsa and hematoxylin and eosin stains were used in processing the tissue sections.

The natural occurrence of antibodies against an enteric virus in some pigs from this herd precluded their use for antiserum production. Therefore, young roosters were utilized. Primary swine kidney cell cultures were not adversely affected by 5 per cent rooster serum and preinoculation samples of rooster serum did not neutralize several selected viral isolates.

Two of the 11 isolates produced type 1 cell destruction and were identified as pleuropneumonia-like organisms (PPLO).[5] Swine given intraperitoneal inoculations of these two PPLO isolates did not develop neutralizing antibodies. No attempt was made to produce neutralizing antibodies against these two isolates in roosters, and neutralization of these two isolates by other antiserum was not done.

A representative isolate of a type 2 cytopathogenic virus (isolate 5) and a type 3 cytopathogenic virus (isolate 9) were selected as antigens. Three roosters were inoculated with undiluted cell-culture fluid containing one of these two viruses. One milliliter of virus suspension was administered intravenously each fifth day and 0.5 ml. was administered intramuscularly each tenth day for a total of 4 intravenous and 2 intramuscular injections. A final inoculation, employing an adjuvant composed of an oil-in-water emulsion of killed *Mycobacterium butyricum* as described by Carpenter,[3] was injected subcutaneously at the rate of 2 ml. divided between two sites. Antiserum collected approximately one week after the last inoculation neutralized $20 \times 10^{6.8}$ and $20 \times 10^{6.5}$ $t.c.i.d._{50}$ (tissue culture infective doses) of homologous virus.

For neutralization trials, tenfold dilutions of virus-containing cell-culture fluid were prepared in Dulbecco's phosphate buffer[2] plus 10 per cent calf serum. An equal amount of a 1:20 dilution of antiserum was added. The mixture was incubated for one hour at 37 C. prior to inoculation. Four cell cultures were inoculated with 0.2 ml. of each preparation. The infective titer of each virus was determined and the difference between the virus titer and the titer of virus plus antiserum was calculated. All viruses having 1 log or more of neutralization were tested against preinoculation samples of rooster serum.

RESULTS

Intranasal inoculation of nine viral and two PPLO nasal isolates into experimental

Received for publication April 15, 1960.

From the Veterinary Medical Research Institute, College of Veterinary Medicine, Iowa State University of Science and Technology, Ames. Published as paper N.S. 612.

This work was supported in part by NC-13 funds.

Dr. L'Ecuyer is an AVMA Research Fellow.

The authors thank Arlene Cole and Janet Welch for technical assistance.

pigs did not produce any clinical evidence of respiratory disease. The pigs inoculated with eight of the nine viral isolates developed mild diarrhea. This finding is not conclusive, since uninoculated control pigs in some groups also developed mild diarrhea. Virus isolate 9 produced clinical illness in inoculated pigs of the first two groups. Two pigs were inoculated intranasally with 0.5 ml. of a sixth-passage cell culture of this virus. One pig developed a febrile reaction on postinoculation (PI) day 6, became incoordinated and had to struggle to rise on PI day 9. On the next day, it was unable to rise and performed running motions. The only lesions found when the pig was necropsied on PI day 10 were petechial hemorrhages on the surfaces of both kidneys and the bladder mucosa. A small area of acute pneumonia was present in the right apical lobe of the lungs. Heart blood, liver, spleen, lungs, and kidneys of this animal yielded no bacterial growth when cultured on blood agar. Lungs, brain, nasal swabs, and a rectal swab were collected from this animal and processed in cell cultures. A type 3 cytopathogenic virus was recovered from only the rectal swab. The second pig was slightly depressed on PI day 19. On PI day 20, the body temperature of this pig was 106.2 F. It returned to normal on day 22, then progressively increased to 105.2 F. on PI day 24. It stayed at about this level until the animal died on PI day 26. At necropsy, this pig had severe peripheral hyperemia of all lymph nodes and petechiae on the surfaces of the kidneys, bladder mucosa, and epiglottis. The lungs were normal in appearance. The peritoneal surface of the diaphragm contained large areas of hemorrhage. There were myocardial hemorrhages, and the skin in the region of the abdomen and ears had numerous discrete hemorrhages. Extreme hemorrhage and edema of the wall of the colon was present. The spleen and kidneys of this second pig were cultured on blood agar, and a pure culture of nonhemolytic *Escherichia coli* was obtained. Nasal and cecal swabs from this animal did not yield a cytopathogenic virus.

A 10-week-old pig was inoculated intraperitoneally with 2 ml. of the same passage of virus 9 as was used to inoculate the previous 2 pigs. On PI day 5, this pig had a body temperature of 105.8 F. A mild diarrhea was present and the animal was moderately depressed. The next day, the pig appeared normal and the body temperature was 104.0 F. The pig was normal at the end of the twenty-two-day observation period. Serum collected at this time neutralized $20 \times 10^{4.5}$ t.c.i.d.$_{50}$ of virus 9 per milliliter of serum. At the end of this 22-day observation period, the pig's immunity was challenged with hog cholera virus. The animal was susceptible, and typical lesions of hog cholera were observed at necropsy.

Four 9-day-old pigs and three 7-week-old pigs were inoculated intranasally; three other 7-week-old pigs were inoculated intraperitoneally with eighth-passage cell culture of virus 9. All of these pigs remained normal. Four additional pigs, 2 weeks old, were inoculated intranasally with virus 9 of the sixteenth passage. These pigs also remained normal. Serum collected 36 days postinoculation from pigs of the latter

TABLE 1—Recovery of Cytopathogenic Pleuropneumonia-like Organisms and Viruses from Experimental Pigs Inoculated Intranasally

Isolate			Postinoculation data					
			First collection			Second collection		
				Results			Results	
Number	Type of cell destruction	Type of agent	Day	Nasal	Rectal	Day	Nasal	Rectal
1	3	virus	7	0/3*	3/3	21	0/3	0/3
2	3	virus	7	1/3	3/3	21	0/3	0/3
3	1	PPLO**	7	0/4	3/4	21	0/3	0/3
4	2	virus	5	0/3	2/3	26	0/3	1/3
5	2	virus	5	0/4	0/4	27	0/4	0/4
6	3	virus	4	2/3	3/3	18	0/3	3/3
7	3	virus	4	2/2	2/2	not done		
8	3	virus	4	2/2	2/2	18	0/2	2/2
9	3	virus	5	0/2	2/2	10	0/1	1/1
9	3	virus	not done			36	0/4	0/4
9	3	virus	not done			36	0/4	0/4
10	2	virus	5	1/2	2/2	28	1/2	2/2
11	1	PPLO	5	2/2	0/2	23	2/2	0/2

* Numerator = number positive; denominator = number of pigs. ** Pleuropneumonia-like organism.

group was found to neutralize $20 \times 10^{1.5}$ t.c.i.d.$_{50}$ of virus 9 per milliliter of serum.

Recovery of the cytopathogenic PPLO and the nine cytopathogenic virus isolates from intranasally inoculated pigs of the various groups is summarized (table 1).

The two PPLO isolates (3 and 11) differed in their site of localization and in the length of time they persisted. Isolate 3 was recovered from rectal swabs on PI day 7 but not on day 21. It was not recovered from nasal swabs. This is the first report of the isolation of PPLO from the swine intestinal tract, although this group of organisms has been recovered from this site in man.[1] Isolate 11 localized in the nasal cavity where it persisted for at least 23 days. It was not recovered from rectal swabs.

Only 1 of 9 pigs given the three viral isolates producing type 2 cell degeneration had virus in the nasal cavity five days postinoculation. This same pig had virus in its nasal cavity when samples were collected again on PI day 28. The viral isolates producing type 2 cell destruction were recovered from the intestinal tracts of 4 of 9 pigs on PI day 5 and from 3 of 9 pigs on PI days 26 to 28.

Four of six viral isolates producing type 3 cell destruction were recovered from the nasal cavities of pigs on PI days 4 to 7. This represents a recovery rate of 7 out of 15 pigs inoculated with these six isolates, none of which persisted in the nasal cavity as long as ten to 26 days postinoculation. These viruses occurred more frequently in the intestinal tracts than in the nasal cavities of inoculated pigs. Virus was recovered from rectal swabs collected on PI days 4 to 7 from all 15 pigs inoculated with these six isolates. This group of viruses was partially eliminated from the intestinal tract of the host, since rectal swabs from only 6 of 20 pigs yielded virus 10 to 36 days postinoculation.

The results of virus neutralization trials with antiserums produced by viral isolate 5 (type 2 cell destruction) and viral isolate 9 (type 3 cell destruction) are summarized (table 2). Variations of less than 1 log in titer were not considered significant. Preinoculation samples of rooster serum did not neutralize any of the viruses neutralized by serum from immune animals.

All three viruses producing type 2 cell destruction were neutralized by antiserum produced against one of them (virus 5). This same antiserum failed to neutralize any of the six viral isolates producing type 3 cell degeneration. Only two of the viral isolates producing type 3 cell destruction were neutralized by the antiserum produced against one of them. This antiserum failed to neutralize any of the viruses producing type 2 cell destruction.

TABLE 2—Neutralization of Type 2 and Type 3 Swine Nasal Viruses by Antiserums Produced Against Two Representative Isolates

Isolate		Titers		
Number	Type of cell destruction	Virus	Virus + isolate 5 antiserum	Virus + isolate 9 antiserum
1	3	$10^{2.5}$*	.....	$10^{2.3}$
1	3	$10^{4.7}$	$10^{5.2}$	.....
2	3	$10^{2.5}$	$10^{2.5}$	$10^{2.0}$
4	2	$10^{1.8}$	$10^{2.0}$	$10^{1.2}$
5	2	$10^{2.0}$	$10^{2.0}$	.....
5	2	$10^{1.5}$	.....	$10^{1.0}$
6	3	$10^{5.2}$	$10^{5.2}$	$10^{5.8}$
7	3	$10^{5.5}$	$10^{5.5}$	$10^{5.5}$
8	3	$10^{4.8}$	$10^{4.8}$	$10^{4.8}$
9	3	$10^{4.8}$	.....	$10^{2.7}$
9	3	$10^{2.5}$	$10^{1.7}$	$10^{0.5}$
10	2	$10^{2.8}$	$10^{0.5}$	$10^{2.5}$

* Fifty per cent tissue culture dose of virus activity per 0.1 ml. of virus, and 0.2 ml. of virus plus an equal amount of a 1:20 dilution of antiserum.

Tissues for histologic examination were collected from the nasal turbinates, tonsils, duodenum, ileum, colon, and certain other organs of several of the experimental pigs. All of the nasal turbinates examined appeared normal, except a specimen from 1 pig inoculated with virus 9 and specimens from 2 of the pigs inoculated with virus 10. In the nasal turbinates of these 3 pigs there was mild lymphocytic infiltration of the submucosa. In addition, a periglandular lymphocytic infiltration of the tubuloalveolar glands was observed.

All tonsils examined were normal, except some of those from pigs inoculated with viruses 7 and 2. Several small foci of degeneration were observed in the lymphoid tissue of these specimens. In addition, virus 7 produced a mild neutrophilic infiltration.

The duodenal specimens examined were normal, except some of those from pigs inoculated with isolates 1, 2, 3, 5, and 9. In these specimens, there was a lymphocytic and plasma cell infiltration of the mucosa and submucosa. In addition, there was marked neutrophilic infiltration of the submucosa in the pigs inoculated with virus 5.

The duodenum of 1 pig given virus 9 had acute inflammation characterized by edema, hyperemia, and necrosis of the villi associated with bacterial invasion. There was serous lymphadenitis in the mesenteric lymph nodes of this animal.

Of the tissues examined from the ileum and colon of most of the animals, all appeared normal except those from pigs given viruses 2 and 9. Pigs inoculated with virus 2 had an eosinophilic infiltration of the submucosae of both the ileum and colon; whereas, the pig inoculated with virus 9 had an eosinophilic reaction in only the ileum.

DISCUSSION

None of the cytopathogenic nasal isolates studied produced any clinical evidence of respiratory disease. Gross lesions of the respiratory tract were not observed, except in 1 pig inoculated with virus 9. Several of the viruses persisted in the nasal cavity for five to seven days after inoculation. The present work does not give adequate insight into the possible occurrence of mild cellular reactions in the nasal turbinates during this period. A definite lymphocytic submucosal infiltration and a periglandular lymphoid infiltration of the nasal turbinates were present in 1 pig ten days after inoculation with virus 9 and in 2 pigs 28 days after inoculation with virus 10. The finding that some of the viruses isolated from the nasal cavities of swine will remain in the nasal cavity several days after inoculation poses an interesting problem as to the possibility that these viruses cause a predisposition to invasion of the nasal cavity by agents causing turbinate atrophy.

The information secured in the present work suggests that the nasal virus isolates have a greater affinity for the intestinal tract than for the nasal cavity, as evidenced by higher incidence of infection and longer period of survival.

The mild diarrhea observed in eight groups of inoculated pigs is not conclusive evidence that some of these agents cause diarrhea, since some uninoculated control pigs also developed diarrhea. One of the two PPLO isolates was recovered from rectal swabs from inoculated pigs. The cytopathogenic agent isolated from the inoculated pigs appeared similar to that used as inoculum. No evaluation of its significance is possible at this time.

There was evidence that a correlation existed between the type of cell destruction produced in cell cultures and the antigenic structure of the viruses causing type 2 cell destruction. The viruses producing type 3 cell destruction appeared to be composed of two or more antigenic groups. The two type 3 viruses that produced microscopic lesions in the ileums of experimental pigs were antigenically similar.

An interpretation of the results observed in pigs inoculated with virus 9 is difficult. It is possible that hog cholera virus was carried through six passages but not eight passages of virus 9. This appears unlikely, since 1 of the pigs inoculated with sixth passage material was susceptible to hog cholera virus. Another possibility is that an attenuation of virus 9 occurred between passages 6 and 8. The most likely possibility is that the pigs in the first two groups were conditioned in some unknown manner so that virus 9 was extremely pathogenic.

SUMMARY

Intranasal inoculation of nine viral and 2 pleuropneumonia-like cytopathogenic isolates from swine nasal cavities failed to produce any clinical evidence of respiratory disease in experimental pigs. Some of the agents were present in the nasal cavities of experimental pigs five to seven days postinoculation. The viral agents were more frequently recovered and persisted for a longer period of time in the intestinal tracts of experimental pigs. One viral isolate produced a febrile reaction or death, or both, in the first two groups of inoculated pigs, but not in pigs of three subsequent groups.

Several of the viral isolates produced mild cellular reaction in the nasal cavities and intestinal tracts of experimental pigs.

Three viral isolates producing one type of cell culture destruction were antigenically similar, but the isolates producing another type of cell culture destruction appeared to have two or more antigenic types.

References

[1] Berg, R. L., Daggett, W., Madden, J., and Dienes, L.: The Origin of PPLO Found in Rectal Cultures. Ann. New York Acad. Sci., 79, (1960): 635–641.

[2] Carpenter, P. L.: Immunology and Serology. W. B. Saunders Co., Philadelphia, (1958): 302–303.

[3] Dulbecco, R. and Vogt, M.: Plaque Formation and Isolation of Pure Lines of Poliomyelitis Virus. J. Exptl. Med., 99, (1954): 167–182.

[4] Switzer, W. P.: Action of Certain Viruses, *Myco. hyorhinis*, and Nasal Trichomonads on Swine Tissue Cultures. Am. J. Vet. Res., 20, (Nov., 1959): 1010–1019.

[5] Switzer, W. P., and L'Ecuyer, C.: Detection of Swine Nasal Viruses in Cell Cultures. Am. J. Vet. Res., 21, (Nov., 1960): 967–970.

SUMMARIO IN INTERLINGUA

Sito de Localisation e Effectos de Virus Nasal Porcin in Porcos Experimental

Le inoculation intranasal de 11 isolatos cytopathogene ab le cavitates nasal de porcos, incluse 9 virusal e 2 pleuropneumonioide, non produceva ulle evidentia clinic de morbo respiratori in porcos experimental. In plure casos il esseva constatate que le agentes esseva ancora presente in le cavitates nasal del porcos experimental post intervallos de cinque a septe dies. Le agentes virusal esseva retrovate plus frequentemente e persisteva plus longemente in le vias intestinal del porcos experimental. Un del isolatos virusal produceva un reaction febril o le morte del animal o ambes in le prime duo gruppos de porcos inoculate sed non in le porcos de tres gruppos subsequente.

Plures del isolatos virusal produceva leve reactiones cellular in le cavitates nasal e le vias intestinal del porcos experimental.

Tres isolatos virusal producente un sol typo de destruction de cytoculturas esseva antigenicamente simile, sed le isolatos producente un secunde typo de destruction cytocultural pareva representar duo o plus typos antigenic.

Post-Tetanic Potentiation in Spinal Cord of Normal and Myoclonic Pigs

M. W. STROMBERG, D.V.M., Ph.D.; R. L. KITCHELL, D.V.M., Ph.D.; R. C. CALLSTROM, D.V.M.

St. Paul, Minnesota

PREVIOUS REPORTS [7,12,13] have dealt with various aspects of a condition in baby pigs characterized by a tremor present at birth. The segmental reflex in the normal pig has been characterized [14] and shown to differ notably from the segmental reflex in pigs affected with tremor. The results further indicated the presence of increased excitability via two-neuron pathways and decreased excitability via multineuronal pathways in pigs with tremor.

The potentiating effect of a burst of "high"-frequency electrical stimulation applied to a dorsal root of the spinal cord, on reflex response to low-frequency stimulation, was first demonstrated in the cat by Woolsey and Larrabee.[17] A similar phenomenon was shown earlier to occur in frogs.[1]

Lloyd [9] has worked out in some detail the effect of post-tetanic potentiation on the monosynaptic reflex and observed that little post-tetanic change attends reflex transmission through polysynaptic reflex arcs.

In experiments performed on anesthetized, decerebrate, and spinal* cats, Ström [11] has attempted to demonstrate a physiologic significance for post-tetanic potentiation. He was unable to obtain potentiation using muscle stretch with its resultant rapid firing of muscle spindle afferents as tetanizing stimulus. Ström therefore concluded that post-tetantic potentiation has little, if any, importance for physiologic forms of spinal reflex transmission.

Granit,[6] however, has shown that tetanization or stretch creates a long-lasting state of hypersensitivity of the ventral horn cells to subsequent stretch reflexes. He has demonstrated the striking similarity between rebound and post-tetanic potentiation and concludes that both phenomena involve the same principles.

Using spinal cats, Wilson [16] has demonstrated that a definite post-tetanic potentiation of polysynaptic reflexes occurs, as well as a monosynaptic potentiation. Most of Wilson's report involves results obtained with respect to the segmental reflex. In most animals tested, the maximum polysynaptic potentiation was obtained with tetanizing frequencies of 25 to 100 shocks per second. Wilson also observed post-tetanic potentiation upon tetanizing a given dorsal rootlet and applying the test shocks to an adjacent dorsal rootlet. Usually the greatest potentiation was seen in the first peak of the polysynaptic response. Hughes [8] has published a review on studies of post-tetanic potentiation.

MATERIALS AND METHODS

The general procedure used was the same as outlined in an earlier report.[14] Three groups of young pigs were used in this study. Group I, composed of 11 pigs having no history of tremor, was used as a control group. Group II consisted of 13 pigs affected with the typical tremor of myoclonia congenita. Group III contained 7 pigs which had recovered from the disease and had no clinical evidence of tremor. Most of the pigs were from 1 to 5 weeks of age. A few of the pigs in groups I and II were up to 4 months of age.

The pigs were anesthetized with intravenously administered 10 per cent allobarbital with urethane.* They were brought to the stage of light surgical anesthesia and maintained at that level throughout the surgical and experimental procedures.

The trachea was cannulated to permit artificial respiration, if needed, and to prevent aspiration of salivary secretions. A laminectomy was performed to expose the sacral and posterior portion

Received for publication March 3, 1960.

From the Division of Veterinary Anatomy, College of Veterinary Medicine, University of Minnesota, St. Paul.

This study was supported by grant No. B-1081 from the National Institute of Neurological Diseases and Blindness, U.S. Public Health Service, Bethesda, Md.

* This term refers to a cat in which the spinal cord has been severed transversely, posterior to the medulla.

* The authors express their appreciation to Ciba Pharmaceutical Products Inc., Summit, N.J., for supplying allobarbital (Dial) with urethane.

of the lumbar spinal cord. The dura mater was split mid-dorsally to expose the dorsal and ventral roots of this region of the spinal cord. The region was immersed in warm (39 C.) mineral oil. The dorsal and ventral rootlets were severed at the point of their penetration of the dura mater and were placed on silver wire electrodes. The stimuli were delivered to the dorsal root by a Grass S-4 B square wave stimulator. The ventral root action potentials were recorded, using an R-C coupled amplifier, a dual-beam cathode-ray oscilloscope, and a recording camera.

A strength of the afferent stimulus which consistently gave maximum segmental reflex response was chosen. The duration of tetanization was arbitrarily set at 15 seconds.

The selection of a tetanization frequency of 250/sec. was based partly on Lloyd and Wilson's [10] work on the effects of varying frequency in this type of experiment. Figure 1 illustrates the plots of a series of post-tetanic potentiations obtained from a control (group I) pig. The duration of tetanization was held constant at 15 seconds, but the frequency was varied as indicated. The tetanization frequency of 250/sec. tended to favor neither the monosynaptic nor the polysynaptic potentiation, whereas subsequently lower frequencies favored the polysynaptic potentiation and higher frequencies favored the monosynaptic potentiation.

Each stimulating and recording procedure began by supramaxillary stimulating the dorsal root at a frequency of one shock per four seconds and recording four to six responses from the ventral root. The stimulus frequency was then changed to 250/sec. and the root tetanized for 15 seconds. The stimulus frequency of one shock per four seconds was then resumed. The entire procedure was recorded photographically until such time as the response returned to near pre-tetanization levels.

In order to reduce the effects of any depression which might follow the post-tetanic potentiation (PTP), at least 15 minutes was allowed to elapse between each tetanization of the dorsal root. Nevertheless, it was frequently noticed that the baseline or control level of the monosynaptic response tended to fall markedly, even after only one PTP trial. Time did not permit an analysis of this effect, but every effort was made to keep the procedure constant for all three groups of pigs.

Description of Post-Tetanic Potentiation (PTP) Results.—Before discussing the specific results in each group of pigs, it is in order to comment on the general effects of dorsal root tetanization in the pig. No difficulty was encountered in obtaining at least some degree of PTP in the ventral root reflex response. As a rule, the degree of potentiation was comparatively great and, in some instances, reached a peak as high as 115 times the pre-tetanization level for the monosynaptic response. The curves of the monosynaptic response all show a fairly sharp rise to a peak value and then a variable rate of decline to a pre-tetanization level (fig. 2, 3).

The most striking feature of the PTP response in pigs appeared to be the comparatively great increase in the polysynaptic reflex response (fig. 2, 3) following tetanization. Since the response was so prominent, the recordings of the ventral root PTP response were analyzed, not only with respect to the monosynaptic spike, but also with respect to the second wave [14] which made up a large part of the polysynaptic response. Since the synchrony of the motor neuron response which went to make up the second wave was, in many instances, as marked as that for the monosynaptic wave, both first and second waves were measured in terms of spike height. Potentiation of the second wave reached eight times control level in 1 animal. The average PTP response of the second wave in control pigs was more than 300 per cent.

All the graphs used to illustrate the PTP results were plotted with absolue values of spike height on the ordinate and time, in seconds, on the abscissa. All of these graphs include response curves for two parts (monosynaptic and second wave) of the ventral root reflex response. One reason for plotting absolute values of spike height rather than multiples of pre-tetanization response was that, frequently, the pre-tetanization value of the monosynaptic response was zero and, therefore, could not be used for a basis of comparison. Another advantage of dealing with absolute values of spike height lies in providing a direct picture of comparative levels of first and second wave response at any given time.

The ratios of maximum first to maximum second wave amplitude following tetanization of the dorsal root have been calculated for each animal, and the averages are listed in table 1. All time values for duration of PTP represent the time elapsed to the point of 70 per cent relaxation from maximum response recorded following the tetanization. The baseline level of reflex response amplitude is the average of four to six ventral root reflex responses recorded immediately before the tetanization. Values of second wave response, enclosed in parentheses ( ), represent duration of depression

TABLE 1—Mean Values for Duration and Amplitude of PTP Response in Three Groups of Pigs

Group	No. of pigs	Av. dur. 1st wave PTP (sec.)*	Range (sec.)	Av. dur. 2nd wave PTP (sec.)	Range (sec.)	1st wave max. / 2nd wave max.	Range
I	11	42	0–69	95	30–218	1.15	0.0–3.22
II	13	94	30–190	39	(194)–174**	2.70	0.28–6.71
III	7	59	18–102	66	12–138	1.18	0.15–1.79

* All duration values are in seconds to 70 per cent relaxation of the post-tetanic potentiation (PTP). Ratios of the first to second wave are in terms of amplitude. ** Values in parentheses ( ) indicate depression.

of this response below control level. These readings also include the time elapsed before return of second wave response 70 per cent of the way back to control level. For example, a (48) reading was calculated as follows: the amplitude value of the lowest second wave response (after tetanization) was subtracted from the average pre-tetanization value. The difference represented 100 per cent depression; 70 per cent of this difference was calculated and the resulting value located on the graph of PTP response. The point in time following tetanus was read off from the graph and, in this instance, the value was 48 seconds.

One reason for utilizing the 70 per cent relaxation value was the impracticability of photographically following the PTP response all the way to control level. For example, in several instances, the total duration of PTP response was as long as eight to ten minutes.

Group I (Control) Pigs.—The PTP response in control pigs was characterized by a consistent and large polysynaptic reflex which, both in terms of magnitude and duration, frequently exceeded the monosynaptic response. Figure 3 illustrates the early portion of a series of photographs obtained during a representative PTP trial. Figure 2 illustrates a plot of a representative PTP response in another pig of this group. The predominance of the polysynaptic reflex is evident. The time to 70 per cent relaxation for the monosynaptic response was 52 seconds. The comparable value for the second wave was 162 seconds.

Table 1 includes the ratios of first wave maximum amplitude to that of second wave maximum amplitude and also includes time values for 70 per cent relaxation of potentiation of both first and second waves in group I pigs.

Group II (Acutely Affected) Pigs.—Figure 3 illustrates photographically and figure 4 graphically the PTP response in a pig with severe tremor involvement at the time of the experiment. One of the response characteristics was the very large monosynaptic wave which gradually fell to a 70 per cent relaxation level over a period of 190 seconds (fig. 4). The potentiation of the polysynaptic response in this pig was very small. The second wave reached a peak of 2.1 times the pre-tetanization level and returned to 70 per cent relaxation only after 192 seconds.

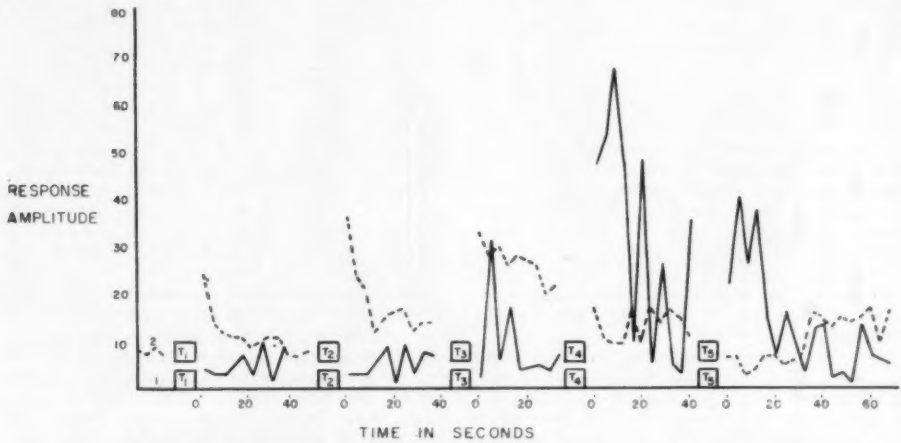


Fig. 1—Variation in post-tetanic potentiation response of first (1————) and second (2------) wave of segmental (S1) reflex as a function of frequency of tetanizing stimulus in a normal pig. $T_1 = 10$/sec., $T_2 = 25$/sec., $T_3 = 250$/sec., $T_4 = 2{,}500$/sec., $T_5 = 10{,}000$/sec.

The average 70 per cent relaxation value for duration of monosynaptic response in group II pigs was 94 seconds (table 1). The duration of second wave potentiation varied considerably in group II pigs, but there was good correlation between the variation and the severity of tremor. For example, the 3 pigs with the most severe tremor had extremely short second wave potentiations.

Reference to table 1 shows the average value of ratio of first to second wave maximums following potentiation in pigs with tremor to be more than twice that of the control and recovered groups. Individual values were well above unity for all but 1 of the 13 animals in this group.

In general, the monosynaptic wave was a prominent part of the potentiated segmental reflex response. The average value for ratio of first to second wave in 13 group II pigs was 2.70, whereas the comparable mean for group I pigs was 1.15 (table 1).

Group III (Recovered) Pigs.—A representative

PTP response in a pig recovered from tremor of myoclonia congenita is illustrated photographically (fig. 3). Another PTP response in a group III pig is graphically shown (fig. 5). Table 1 includes the 70 per cent relaxation values for the duration of PTP response in this group of pigs, also. On this basis, the mean value for duration of monosynaptic potentiation in all 7 pigs was 59 seconds. Mean duration of second wave potentiation was 66 seconds.

Discussion

The hyperactive monosynaptic and hypoactive polysynaptic response noticed previously [14] with respect to the segmental reflex in pigs with tremor is further emphasized after dorsal root tetanization.

In some of the pigs with tremor, there was an actual depression of the polysynaptic response following dorsal root tetanization. None of the control or recovered animals had this type of response.

Both duration and magnitude of monosynaptic response following afferent tetanization were markedly greater in pigs with tremor as compared to control pigs (table 1). Recovered pigs had PTP response characteristics which fell between those of normal and affected animals. The reason for this may be that some of the recovered pigs had only recently ceased to have signs of tremor. It should be emphasized also that there was considerable variation in severity of tremor in group II pigs; this might explain the rather broad ranges of experimental values obtained.

Lloyd's[9] report on post-tetanic potentiation emphasizes the insignificance of polysynaptic potentiation, whereas Wilson [16] found that, with comparatively low stimulation frequencies, a marked polysynaptic potentiation was observed. The results of our work on pigs agree essentially with those of Wilson. However, low tetanization frequencies (25/sec.) were not at all essential to obtaining this effect. Polysynaptic responses of two to five times the pre-tetanization levels were routinely obtainable in control and recovered pigs. Also in agreement with Wilson's [16] observation was the tendency for the polysynaptic response to become more synchronous following afferent nerve tetanization.

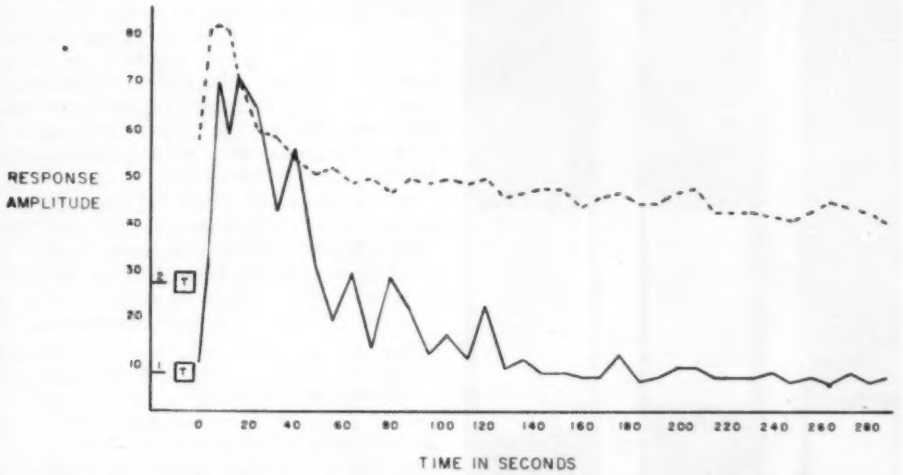


Fig. 2—Post-tetanic potentiation response of monosynaptic (1————) and second wave (2------) in normal pigs. Graph represents three trials. T = 15 seconds of stimulation at 250 shocks per second.

Clearly, the monosynaptic response, as typically elicited experimentally, can best be interpreted as a test of ability of the lower motor neurons to respond to spatial summation. Previously, it might have been argued that such a test would be complicated by inhibitory effects of afferent nerve stimulation. Recently, however, it has been

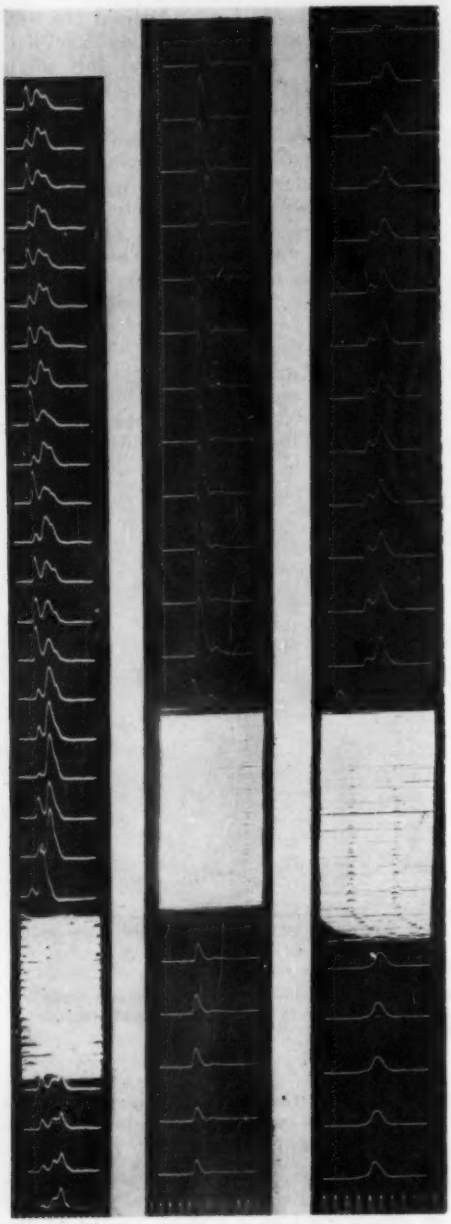


Fig. 3—Post-tetanic potentiation response in 1 pig from each of the three groups. Stimulation dorsal root segment 1, recorded ventral root segment 1. Time is in milliseconds.

demonstrated [4] that inhibitory arcs involve at least one interneuron. This leaves the monosynaptic response purely as an indicator of the excitatory effect of impulses traversing the fast afferents from the muscle spindles. However, this statement can approach accuracy only if the stimuli are spaced far enough apart to avoid residual effects both from lower motor neuron (LMN) and interneuron activity. Frank and Fuortes [5] have shown that interneurons may fire repetitively for as long as one second. Lloyd and Wilson [10] have shown that there may be some monosynaptic depression if the stimulating frequency is greater than three per minute.

It is doubtful that the monosynaptically fired LMN's can be considered as a "race to themselves." Suda *et al.*[15] have shown that a given LMN can be made to fire either "monosynaptically" or "polysynaptically." Thus, at a high level of excitability, the LMN can respond to the spatial summation of impulses necessary to produce the monosynaptic response. At a lower level of excitability, the myriad pulsations of the interneurons can play a part (both excitatory and inhibitory) in determining whether the LMN shall fire.

When one considers the "un-physiologic" nature of the stimulus used to elicit the monosynaptic response, it seems futile to attempt to ascribe a function to it. Rather, it seems better simply to consider it as a test for response to spatial summation.

In any discussion of tremor, it would appear to be true that a high degree of synchrony of synergist-contraction is involved. If the asynchronous activity of motor units (which lies at the basis of normal tone) were to be present, it is difficult to see how tremor could also exist. The alternating synchronous contractions of agonist group vs. antagonist group (not necessarily extensors vs. flexor) may be difficult to detect when observing a fine tremor but is obvious in the 2 to 3 per second "jumping" activity of some myoclonic pigs.[12]

For a tremor to be maintained reflexly (viz., limb tremor in a standing animal), it would seem necessary that the reflex response to stretch should irradiate widely and synchronously in a given group of synergistic muscles. Thus, a muscle group (such as the flexors) would contract to-

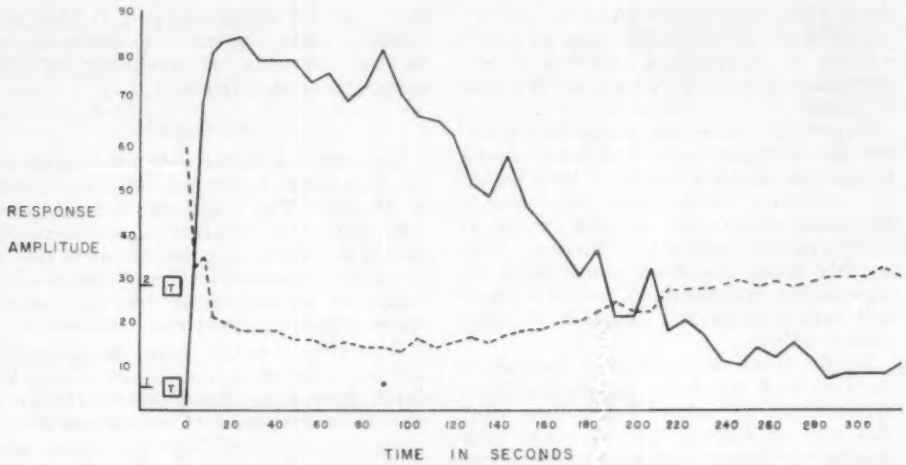


Fig. 4—Graph of post-tetanic potentiation response of monosynaptic (1————) and second wave (2 - - - - - -) of segmental reflex in a pig with tremor—three trials. T = 15 seconds stimulation at 250 shocks per second.

gether and, by so doing, reflexly stimulate the extensors as a group.

Any tendency of the LMN's to fire monosynaptically and synchronously reduces the influence of the prolonged interneuronal activity which could maintain a smooth tonic contraction of the muscle. In other words, flexors and extensors are alternating in phasic contraction.

Physiologically, the sensory input to the spinal cord from muscles has been shown to consist mainly of the fairly high-frequency impulses originating in muscle spindles and tendon organs. If the state of excitability of the LMN is such that it responds directly (monosynaptically) to this afferent input (as reflected by predominance of monosynaptic response in myo-

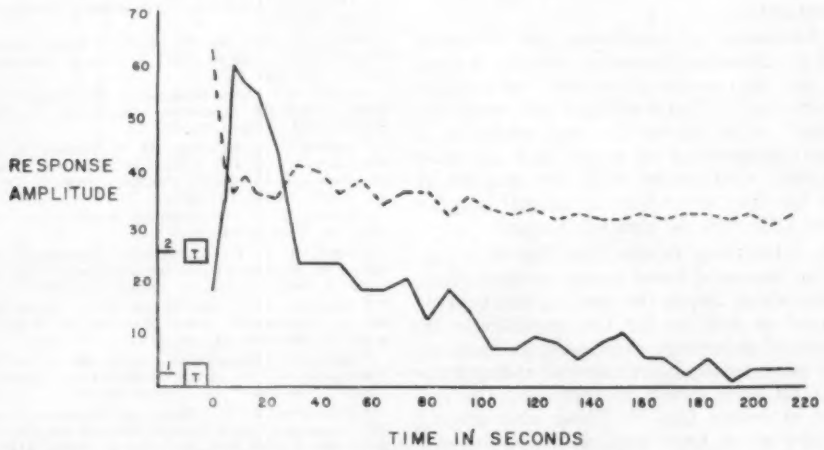


Fig. 5—Graph of post-tetanic potentiation response of monosynaptic (1————) and second wave (2 - - - - -) response in a group III (recovered) pig. T = 15 seconds stimulation at 250 shocks per second.

clonic pigs), it is far less under the stabilizing influence of the long-lasting repetitive activity of interneurons and thus is governed mainly by its own natural frequency of firing.

This theory does not necessarily postulate any decreased activity of interneurons. It could be simply a matter of their having no "substrate" to act upon, since most of the motor neuron pool is being excited directly (monosynaptically). However, there is little doubt that even under these circumstances interneuronal activity would still have a modifying influence on motor neuron output.

Single shock stimulation [14] has shown the LMN's of myoclonic pigs to be hyperexcitable to the spatial summation which this sort of testing involves. The long-lasting PTP response in myoclonic pigs reveals the susceptibility of the LMN to temporal summation of the sort involved in normal physiologic activity.[6]

Location of the exact site of abnormality is admittedly difficult. Work by Campbell [2] and Downman *et al.*[3] has shown that the reflex response from chromatolysed motor neurons is lacking in a monosynaptic component. The large monosynaptic response and the lack of any histologically demonstrable change* in the spinal cord of myoclonic pigs seems to eliminate the spinal cord as a site of abnormality. Actual demonstration of the primary site of abnormality remains a problem for future investigation.

Observation of trembling pigs indicates that the abnormal muscular activity is present only in muscles which are "voluntarily innervated." This is evident both while the animal is in locomotion and while it is simply supporting its weight in a standing position. Ordinarily, with the muscles at rest (as they are when the animal is lying down), there is no sign of tremor.

It is tempting to speculate that it is simply an increased lower motor neuron excitability which lies at the root of this type of tremor as well as for the physiologic response of shivering. Cold, pain, excitement, and parenterally administered epinephrine have all been shown to aggravate the tremor in intact pigs.[12] These also are all factors which have been shown to increase motor neuron excitability and, further, are capable, under certain circumstances, of causing shivering or trembling in supposedly normal animals.

* Stromberg, M. W., St. Paul, Minn.: Unpublished observations.

SUMMARY

The effect of dorsal root tetanization on the first sacral segmental reflex was tested in 31 pigs. The responses were analyzed with respect to duration of monosynaptic and of second wave potentiation to the 70 per cent relaxation level and comparative maximum amplitudes of first and second waves following dorsal root tetanization.

With respect to the above characteristics, marked differences were demonstrated between control pigs and pigs with tremor of myoclonia congenita. Less marked differences were observed between control pigs and recovered pigs.

The nature of the spinal cord reflex response in pigs with tremor suggests increased excitability via two-neuron-arc pathways and decreased excitability via multi-neuronal pathways.

References

[1] Bremer, F., and Kleyntjens, J.: Étude du phenomène de la facilitation centrale. Ann. Physiol. Physiochem. Biol., 10, (1934): 874–878.

[2] Campbell, B.: The Effects of Retrograde Degeneration Upon Reflex Activity of Ventral Horn Neurons. Anat. Rec., 88, (1944): 25–35.

[3] Downman, C. B. B., Eccles, J. C., and McIntyre, A. K.: Functional Changes in Chromatolysed Motoneurons. J. Comp. Neurol., 98, (1953): 9–36.

[4] Eccles, E. M., and Lundberg, A.: The Synaptic Linkage of "Direct" Inhibition. Acta physiol. Scandinav., 43, (1958): 204–215.

[5] Frank, K., and Fuortes, M. F.: Unitary Activity of Spinal Interneurons of Cats. J. Physiol. London, 131, (1956): 424–435.

[6] Granit, R.: Reflex Rebound by Post-Tetanic Potentiation. Temporal Summation—Spasticity. J. Physiol. London, 131, (1956): 32–51.

[7] Hanson, L. J., Stromberg, M. W., Kitchell, R. L., and Kernkamp, H. C. H.: Studies on Myoclonia Congenita. II. Gross and Microscopic Pathology. Am. J. Vet. Res., 19, (April, 1958): 383–387.

[8] Hughes, J. R.: Post-Tetanic Potentiation. Physiol. Rev., 38, (1957): 91–113.

[9] Lloyd, D. P. C.: Post-Tetanic Potentiation of Response in Monosynaptic Reflex Pathways of the Spinal Cord. J. Gen. Physiol., 33, (1949): 147–170.

[10] Lloyd, D. P. C., and Wilson, V. J.: Reflex Depression in Rhythmically Active Monosynaptic Reflex Pathways. J. Gen. Physiol., 40, (1957): 409–425.

[11] Ström, G.: Physiological Significance of Post-Tetanic Potentiation of the Spinal Monosynaptic Reflex. Acta physiol. Scandinav., 24, (1951): 61–83.

[12] Stromberg, M. W.: Studies on Myoclonia Congenita. III. Drugs and Other Factors Affecting Severity of Tremor. Am. J. Vet. Res., 20, (March, 1959): 319–323.

[13] Stromberg, M. W., and Kitchell, R. L.: Studies on Myoclonia Congenita. I. Review of Literature and Field Investigations. Am. J. Vet. Res., 19, (April, 1958): 377–383.

[14] Stromberg, M. W., and Kitchell, R. L.: Studies on Myoclonia Congenita. IV. The Segmental Reflex in Normal and Affected Pigs. Am. J. Vet. Res., 20, (July, 1959): 627–633.

[15] Suda, I., Koizumi, K., and Brooks, C. M.: Reticular Formation Influences on Neurons of Spinal Reflex Pathway. J. Neurophysiol., 21 (1958): 113–123.

[16] Wilson V. J.: Post-Tetanic Potentiation of Polysynaptic Reflexes of the Spinal Cord. J. Gen. Physiol., 39, (1955): 197–206.

[17] Woolsey, C. N., and Larrabee, M. G.: Potential Changes and Prolonged Reflex Facilitation Following Stimulation of Dorsal Spinal Roots. Am. J. Physiol., 129, (1940): 501–502.

SUMMARIO IN INTERLINGUA

Potentiation Post-Tetanic in le Medulla Spinal de Porcos Normal e de Porcos Myoclonic

Le effecto de tetanisation de radice dorsal super le reflexo del prime segmento sacral esseva testate in 31 porcos. Le responsas esseva analysate con respecto al duration del potentiation monosynaptic e del potentiation del secunde unda usque al nivello de relaxation a 70 pro cento e al amplitudes maximal del prime e secunde undas post tetanisation de radice dorsal.

Con respecto al supra-mentionate characteristicas, marcate differentias esseva demonstrate inter porcos de controlo e porcos con tremor de myoclonia congenite. Minus marcate differentias esseva observate inter le porcos de controlo e porcos restablite.

Le natura del responsa reflexe del medulla spinal in porcos con tremor suggere un augmento del excitabilitate per vias a arco bi-neuronal e un reduction del excitabilitate per vias multi-neuronal.

Correlation of the Effects of Parathion on Erythrocyte Cholinesterase with Symptomatology in Pigs

W. T. OLIVER, D.V.M., M.Sc., Ph.D., and H. S. FUNNELL, B.A.

Guelph, Ontario

THE ABILITY of many of the organophosphate insecticides, both singly and in combination, to depress cholinesterase activity has been well established, and the pharmacologic effects and lethal doses of a number of the economically important members of the group have been described for various animal species.[2, 7-14, 16-20, 23, 24, 26-28, 31-34] Although several of the organic phosphate esters have been reported to cause moderate to pronounced anesthesia and respiratory depression, independent of their anticholinesterase activity,[27, 31] it has been shown that most of the toxic effects produced by members of the group appear to depend upon their ability to inhibit cholinesterase.[9-12, 18, 24-26, 30, 31] However, it has been found that, under field conditions, the degree of enzyme inhibition has not always reflected the severity of the clinical signs.

In the present study, the relationship of symptomatology to the rate and degree of blood cholinesterase inhibition has been investigated. In the intact animal, the blood cholinesterases are exposed earlier to larger concentrations of its inhibitor than are the esterases of other tissues; thus, the degree of inhibition of the blood cholinesterases need not reflect that in other sites. Since the inhibitory effect of parathion on brain cholinesterase had been shown [18] to parallel that in blood, this compound was selected as the anticholinesterase agent.

The first *in vitro* studies on the cholinesterase-inhibiting action of this compound were reported by Dubois *et al.*[13] Diggle and Gage [6] reported that, although purified parathion had a low *in vitro* inhibitory effect, it is highly toxic to animals, suggesting that it is converted *in vivo* to some more active cholinesterase inhibitor. Aldridge and Davison [1] showed that, at least *in vitro*, its oxygen analogue, paraxon, could fill this role.

Received for publication Jan. 15, 1960.

From the Department of Physiological Sciences, Ontario Veterinary College, Guelph, Ont., Canada.

The authors thank G. Smeenk for technical assistance.

MATERIALS AND METHODS

Pretreatment cholinesterase determinations were made on the erythrocytes and plasma of each experimental animal by the method of Fleischer and Pope,[15] using heparin as the anticoagulant. The colored complex developed by ferric chloride was read in a Klett-Summerson colorimeter, using a No. 54 filter. Blood samples were taken from each individual at least once a week after the beginning of drug administration.

A technical grade preparation containing 15 per cent parathion was dispensed daily into gelatin capsules and administered orally.

A preliminary trial was carried out using a single 10-week-old pig to estimate a level of parathion which may produce a continuous, nonacute depression of the red blood cell cholinesterase activity (fig. 1).

Six 10-week-old Yorkshire littermates were allotted to two groups. Three pigs (group A) were given 1.7 mg. of parathion per kilogram of body weight per day as a basic dose.

Because the pigs in this group were being used during their active growing phase, the size of the dose was adjusted at ten-day intervals in order to compensate for the increasing body weight. To ensure progressive enzyme inhibition, beginning on the fifteenth day and at ten-day intervals, the total dose was increased at the rate of 1 mg. of parathion per day of exposure.

The other 3 pigs (group B) were given a single oral dose of 85 mg./kg., calculated as parathion.

RESULTS

ERYTHROCYTE CHOLINESTERASE INHIBITION AND RECOVERY

Preliminary Experiment.—The erythrocyte cholinesterase activity of the pig (fig. 1) decreased during 12 days in response to the initial level of 0.85 mg./kg./day of parathion. In spite of continued exposure to parathion, the degree of cholinesterase inhibition was only slightly more suppressed between days 12 and 17, so the rate of administration was increased to 1.0 mg./kg./day. Activity was more acutely depressed by day 22. Since there was no reason to presume that the rate of enzyme inhibition at this dose exceeded the rate of erythrocyte regeneration, the parathion intake was increased on day 25. However,

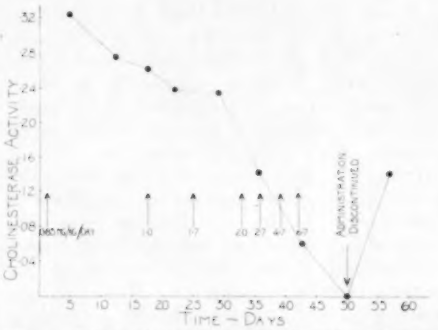


Fig. 1—Effect of the daily administration of parathion on the erythrocyte cholinesterase activity of pig 899. Activity is expressed as a measure of the number of micromoles of acetylcholine hydrolyzed by the blood in 10 minutes at 25 C.

from the results obtained on day 29, it was apparent that cholinesterase activity was leveling off again. To produce progressive inhibition at a rate approaching maximum, the drug was administered in increasing doses beginning on day 33 and at three-day intervals thereafter.

Under these conditions, the activity fell consistently and, on day 50, we were unable to detect any cholinesterase activity in the red blood cells of the animal.

Recovery of activity was most marked during the early phase of the post-treatment period; 39 per cent of the pretreatment average had been regained by the seventh day after the final dose of parathion and only 41 per cent more in an additional 44 days.

Group A.—The cholinesterase activity remained within the pretreatment range of the individual for the first eight days of administration, then was gradually suppressed and reached its lowest activity between days 49 and 52 (fig. 2). Although the cholinesterase activity of any 1 pig decreased irregularly during the period of exposure to the organophosphate, the average activity of the group was consistently inhibited.

The recovery of cholinesterase activity was most rapid during the period immediately following the removal of parathion. It required approximately three days after the last exposure for an average return of 25 per cent of enzyme activity, 14 days for a return of 50 per cent of the activity, 35 days for 75 per cent activity, and 55 days for 90 per cent.

Group B.—One pig died before a blood sample was obtained and the only observation was made on the 2 remaining members of this group at six hours after parathion administration At this time, 19 per cent of the pretreatment red blood cell cholinesterase activity remained in pig 62 and 23 per cent in pig 63.

PLASMA CHOLINESTERASE INHIBITION AND RECOVERY

Preliminary Experiment.—The plasma cholinesterase activity increased slightly during the first 29 days of exposure to parathion. It then decreased and reached its lowest detected value, 58 per cent of the pretreatment activity, on day 50.

The rate of recovery was rapid. Activity returned to within the pretreatment range seven days after parathion administration was discontinued, was well above the original maximum on the eleventh day, but had stabilized within previous limits by the sixteenth day.

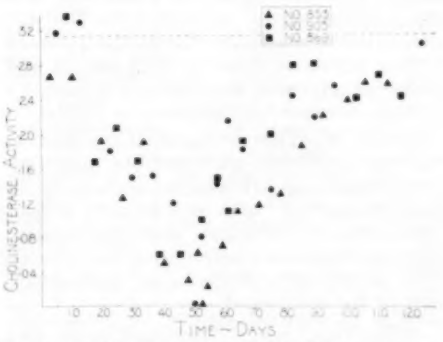


Fig. 2—Effect of the daily administration of parathion on the erythrocyte cholinesterase activity of members of group A and the recovery following discontinuation of the drug.

Group A.—During the first three weeks of daily exposure to parathion, the average response of plasma cholinesterase activity was to increase (fig. 3) toward the upper limit of the pretreatment activity range. After day 25, activity was depressed and on day 38 it fell, remaining below the lower pretreatment limit. The maximum inhibition was produced between days 43 and 53. Activity increased sharply immediately following termination of exposure. It then fluctuated, but returned and remained above the lower limits of the pretreatment

range after day 21 and reached a second apparent maximum on about day 42.

Group B.—Six hours after administration of the single dose of parathion, the cholinesterase activity of the plasma was reduced to 51 per cent of the pretreatment activity in pig 63 and to 30 per cent in pig 62. Death prevented further analyses.

CLINICAL SIGNS

Preliminary Experiment and Group A.—No sign of organophosphate toxicosis was observed in pig 899 at any stage of exposure or after parathion administration was discontinued, nor was there any apparent change in the function or behavior of any group A pig for 48 days. On day 49, the appetite of pig 853 was depressed, but incoordination, muscular tremors, or reluctance to move were not obvious. On the following day, fine muscle tremors were observed and the animal was slightly incoordinated, but the other 2 pigs had neither central nor peripheral disturbance. Administration of parathion was discontinued at the onset of signs, and the mild skeletal muscle effects disappeared within 24 hours. Growth, both during and following exposure at this level, followed a pattern normal for this species (table 1).

Group B.—The course of the poisoning was rapid. Within six hours, the pigs were incoordinate, and there was dyspnea, moderate salivation, and pronounced miosis. The signs of muscular atonicity became progressively more severe and the animals died within eight hours after administration of the compound.

DISCUSSION

In swine, the decrease in the activity of erythrocyte cholinesterase occurred earlier than that of plasma, thus was unlike the effects reported following the continuous exposure of dogs to 500 p.p.m. of 0,0-dimethyl 2:2:2 trichloro-1-hydroxyethylphosphonate* in the ration,[34] in which the fall in plasma cholinesterase was more rapid than that of the erythrocytes. However, the graphs presented by these workers indicated that this difference between the responses of plasma and erythrocyte cholinesterases was due to dosage rather than to some characteristic effect of the drug.

* Dipterex, Chemagro Corp., Kansas City, Mo.

It has been reported that the activity of erythrocyte cholinesterase reached an equilibrium well above zero when rats[4] and dogs[5] were exposed repeatedly to sarin and tabun. The data obtained in the present study suggested that a similar equilibrium can result following the chronic exposure of pigs to parathion. Since the inactivation of the enzyme by parathion is reversible,[3,18] the equilibrium was accounted for in part by red blood cell regeneration and in part by dissociation of the enzyme-parathion complex. However, the state was readily overcome by increasing the rate of parathion administration, and the degree of inhibition became progressively acute until no activity could be detected.

TABLE 1—Effects of Chronic Exposure to Parathion on the Growth of Pigs in Group A

Animal No.	Initial weight (lb.)	Final weight (lb.)	△ Wt. (lb.)	△ T (days)	△ Wt./ △ T
853	35	198	163	124	1.60
905	38	245	207	124	1.67
869	37	236	197	124	1.59

The recovery of red blood cell cholinesterase activity in pigs was diphasic and consisted of a primary phase, in which regeneration was rapid, and a secondary, slow, recovery phase. It required three days for a group average recovery of 25 per cent, but almost five times as long for the next 25 per cent and 12 times for the recovery of the third quartile. The rate of gain in this species was similar to that reported for human beings poisoned by parathion[22]; their red blood cell activity returned at an average of 10 per cent per day for the first three days and one to two per cent per day thereafter. In a study of the cholinesterase recovery mechanism following organophosphate poisoning, Blaber and Creasey[3] considered that the rapid first stage of recovery was related chiefly to the spontaneous hydrolysis of the inhibited enzyme and that the slower second stage was correlated with the synthesis of new enzyme.

The early response pattern of plasma cholinesterase to parathion differed considerably from that of the erythrocytes. In studies on feeding organophosphates to dogs and rats, Frawley and co-workers[16,18] observed an increase in plasma cholinesterase above the levels of the control animals

and termed this compensatory effect, "rebound phenomenon." During the first nine weeks of administration of parathion, Rider *et al.*[29] also reported a moderate increase (4%) in the cholinesterase level of human plasma, which they considered to be due to the effects of this rebound mechanism. A curve, fitted by inspection to the data in figure 3, showed that the average response of plasma cholinesterase of pigs similarly tended to increase moderately during the first few weeks of exposure, reaching a maximum by about the 28th day; thereafter, activity was continuously depressed, reaching an average minimum by about the 50th day. Complete inhibition was not detected in the plasma of any of these animals. In the case of pig 905, having reached a minimum above zero, the plasma cholinesterase activity was not further suppressed by the continuing daily administration of parathion for seven days; this resembled observations previously made in dogs.[34] Unlike the effects following chronic exposures of human beings to malathion,[21] the plasma cholinesterase of the pigs in group A increased sharply following the removal

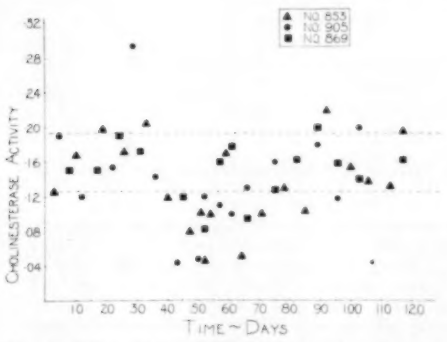


Fig. 3—Effect of the daily administration of parathion on the plasma cholinesterase activity of members of group A and the recovery following discontinuation of the drug.

of parathion and a large part of the activity had been recovered within the first few days. Plasma cholinesterase activity increased moderately about the 42nd day after cessation of treatment. However, both this increase and that observed during the first four weeks of the experiment were slight, barely exceeding the pretreatment maximums (fig. 3).

Summary

In this study, the production of clinical signs by parathion in swine was dependent upon the rate and degree of depression of erythrocyte cholinesterase activity rather than primarily upon the degree of inhibition. Chronic parathion poisoning caused complete inhibition of erythrocyte cholinesterase without a necessary concurrent skeletal muscle paralysis; of the 4 pigs used, only 1 was mildly affected, although erythrocyte cholinesterase activity had been completely inhibited in at least 3 of them. Conversely, acute poisoning caused a rapid depression of erythrocyte cholinesterase and produced acute paralytic signs in all members of the group without complete inhibition of erythrocyte cholinesterase. Neither the acute nor chronic effects of the compound on plasma cholinesterase could be linked in any way with the clinical signs.

References

[1] Aldridge, W. N., and Davison, A. N.: The Inhibition of Erythrocyte Cholinesterase by Tri-Esters of Phosphoric Acid. 2. Diethyl p-Nitrophenyl Thionphosphate (E605) and Analogues. Biochem. J., 52, (1952): 663.

[2] Bagdon, R. E., and DuBois, K. P.: Pharmacologic Effects of Chlorthion, Malathion and Tetrapropyl Dithionopyrophosphate in Mammals. Arch. internat. pharmacodyn., 103, (1955): 192.

[3] Blaber, L. C., and Creasey, N. H.: The Recovery of Brain and Erythrocyte Cholinesterase Activity Following Organophosphate Poisoning. Biochem. J., 71, (1959): 7.

[4] Cohen, B. S., Oberst, F. W., Crook, J. W., and Harris, D.: Effect of Repeated Exposures of G. B. Vapor on Erythrocyte and Brain Cholinesterases in Rats. J. Pharmacol. & Exptl. Therap., 116, (1956): 209.

[5] Craig, F. N.: Canine Erythrocyte Cholinesterase Activity in Chronic Tabun Intoxication. J. Pharmacol. & Exptl. Therap., 126, (1959): 174.

[6] Diggle, W. M., and Gage, J. C.: Cholinesterase Inhibition *in Vitro* by 0,0-Diethyl 0-p-nitrophenyl Thiophosphate (Parathion, E605). Biochem. J., 49, (1951): 491.

[7] Dougherty, E.: Thiophosphate Poisoning in White Pekin Ducks. Avian Dis., 1, (1957): 127.

[8] DuBois, K. P.: Potentiation of the Toxicity of Insecticidal Organic Phosphates. Am. M. A. Arch. Indust. Health, 18, (1958): 488.

[9] DuBois, K. P., Thrush, D. R., and Murphy, S. D.: Studies on the Toxicity and Pharmacologic Actions of the Dimethoxy Ester of Benzotriazine Dithiophosphoric Acid (DBD, Guthion). J. Pharmacol. & Exptl. Therap., 119, (1957): 208.

[10] DuBois, K. P., and Cotter, G. J.: Studies on the Toxicity and Mechanism of Action of Dipterex. Am. M. A. Arch. Indust. Health, 11, (1955): 53.

[11] DuBois, K. P., and Coon, J. M.: Toxicity of Organic Phosphorus Containing Insecticides to Mammals. Am. M. A. Arch. Indust. Hyg., 6, (1952): 9.

[12] DuBois, K. P., Doull, J., Deroin, J., and Cummings, O. K.: Studies on the Toxicity and Mechanism of Action of Some Insecticidal Thionophosphates. Am. M. A. Arch. Indust. Hyg., 8, (1953): 350.

[13] DuBois, K. P., Doull, J., Salerno, P. R., and Coon, J. M.: Studies on the Toxicity and Mechanism of Action of p-Nitrophenyl Diethyl Thionophosphate (Parathion). J. Pharmacol. & Exptl. Therap., 95, (1949): 79.

[14] Durham, W. F., Gaines, T. B., and Hayes, W. J.: Paralytic and Related Effects of Certain Organic Phos-

phorus Compounds. Am. M. A. Arch. Indust. Health, 13, (1956): 326.

[15] Fleisher, J. H., and Pope, E. J.: Colorimetric Method for Determination of Red Cell Cholinesterase Activity in Whole Blood. Am. M. A. Arch. Indust. Hyg., 9, (1954): 323.

[16] Frawley, J. P., and Fuyat, H. N.: Effect of Low Dietary Levels of Parathion and Systox on Blood Cholinesterase in Dogs. J. Agric. & Food Chem., 5, (1957): 346.

[17] Frawley, J. P., Fuyat, H. N., Hagan, E. C., Blake, J. E., and Fitzhugh, O. G.: Marked Potentiation in Mammalian Toxicity from Simultaneous Administration of Two Anticholinesterase Compounds (E.P.N.). J. Pharmacol. & Exptl. Therap., 121, (1957): 96.

[18] Frawley, J. P., Hagan, E. C., and Fitzhugh, O. G.: A Comparative Pharmacological and Toxicological Study of Organic Phosphate Anticholinesterase Compounds. J. Pharmacol. & Exptl. Therap., 105, (1952): 156.

[19] Frawley, J. P., Hagan, E. C., Fitzhugh, O. G., Fuyat, H. N., and Jones, W. I.: Marked Potentiation in Mammalian Toxicity from Simultaneous Administration of Two Anticholinesterase Compounds. J. Pharmacol. & Exptl. Therap., 119, (1957): 147.

[20] Furman, D. P.: Toxicity of Malathion to Poultry and Their Ectoparasites. J. Econ. Entomol., 49, (1956): 447.

[21] Goltz, H. H.: Controlled Human Exposure to Malathion Aerosols. Am. M. A. Arch. Indust. Health, 19, (1959): 516.

[22] Grob, D., Garlick, W. L., and Harvey, A. M.: Toxic Effects in Man of Anticholinesterase Insecticide Parathion (p-nitrophenyl diethyl thionophosphate). Bull. Johns Hopkins Hosp., 87, (1950): 106.

[23] Hagan, E. C., and Woodward, G.: Toxicity of 0,0-diethyl 0-p-nitrophenyl thiophosphate (Parathion). Fed. Proc., 7, (1948): 224.

[24] Hazelton, L. W., and Holland, E. G.: Toxicity of Malathion. Am. M. A. Arch. Indust. Hyg., 8, (1953): 399.

[25] Kewitz, H.: Reactivation of Inhibited Cholinesterase by a Specific Antidote to Alkyl Phosphate Poisoning. Klin. Wchnschr., 35, (1957): 521.

[26] Mangun, G. H., and DuBois, K. P.: Toxicity and Mechanism of Action of Tetraethyl Pyrophosphate. Fed. Proc., 6, (1947): 353.

[27] Pochet, A.: Effects of Neurotoxic Organic Phosphorus Compounds on Respiration. Acta belg. Arte méd. pharm. milit., 3, (1957): 153.

[28] Radeleff, R. D., and Woodward, G. T.: The Toxicity of Organic Phosphorus Insecticides to Livestock. J.A.-V.M.A., 130, (March 1, 1957): 215–216.

[29] Rider, J. A., Moeller, H. C., Swaber, J., and Weilerstein, R. W.: The Effect of Parathion on Human Red Blood Cell and Plasma Cholinesterase. Am. M. A. Arch. Indust. Health, 18, (1958): 442.

[30] Schweitzer, A., Stedman, E., and Wright, S.: Central Action of Anticholinesterases. J. Physiol., 96, (1939): 302.

[31] Stewart, W. C.: The Effects of Sarin and Atropine on the Respiratory Centre and Neuromuscular Junctions of the Rat. Canad. J. Biochem., 37, (1959): 651.

[32] Vandekar, M.: Anaesthetic Effect Produced by Organophosphorus Compounds. Nature, 179, (1957): 154.

[33] Wilber, C. G., and Morrison, R. A.: The Physiological Action of Parathion in Goats. Am. J. Vet. Res., 16, (April, 1955): 308–313.

[34] Williams, M. W., Fuyat, H. N., and Fitzhugh, O. G.: The Subacute Toxicity of Four Organic Phosphates to Dogs. Toxicology, 1, (1959): 1.

SUMMARIO IN INTERLINGUA

Correlation del Effectos de Parathion Super le Cholinesterase Erythrocytic con le Symptomatologia in Porcos

In le hic-reportate studio, le production de signos clinic per parathion in porcos dependeva del intensitate e del grado del depression in le activitate de cholinesterase erythrocytic plus tosto que primarimente del grado de inhibition. Chronic invenenamento per parathion causava le complete inhibition de cholinesterase erythrocytic, sin le occurrentia necessari de un concomitante paralyse skeleto-muscular. Inter le 4 porcos usate, solo 1 esseva levemente afficite, ben que le activitate de cholinesterase erythrocytic habeva essite inhibite completemente in al minus 3. Del altere latere, un invenenamento acute causava le rapide depression de cholinesterase erythrocytic e produceva acute signos paralytic in omne le membros del gruppo, e isto sin le complete inhibition de cholinesterase erythrocytic. Ni le acute ni le chronic effectos del composito super le cholinesterase del plasma poteva esser relationate in ulle maniera con le signos clinic.

The Histopathologic Characteristics of Diseases Producing Inflammation of the Air Sacs in Turkeys—A Comparative Study of Pleuropneumonia-like Organisms and Ornithosis in Pure and Mixed Infections

J. N. BEASLEY, D.V.M., M.S.; R. W. MOORE, D.V.M., M.S.;
J. R. WATKINS, D.V.M., M.S.

College Station, Texas

THE PUBLIC HEALTH implications of ornithosis infection in the turkey have emphasized the need of differentiating this disease from others characterized by similar lesions. In a recent study of the lesions produced in turkeys by the ornithosis agent,[2] many features of the inflammatory process in the respiratory system were found to be similar to those occurring in infectious sinusitis. Because pleuropneumonia-like organisms (PPLO),* which cause infectious sinusitis are transmitted through the egg and, thus, are widely dispersed through the turkey population,[13] there exists the possibility of PPLO infection occurring concomitantly with ornithosis. It was, therefore, considered desirable to study, histopathologically, the lesions produced by these agents in both pure and mixed infections.

In infectious sinusitis, the histopathologic changes are characterized by lymphofollicular and exudative-granulomatous reactions.[4,7,8,10-12,21] The lumina of infected sinuses contain gelatinous or caseous exudates, while in the mucosa, epithelial thickening and degeneration are accompanied by infiltration of lymphocytes and heterophils. Similar changes occur in the mucosa of the trachea. The pneumonic lesions have been referred to as bronchopneumonia or exudative-granulomatous pneumonia. Inflammatory changes in the air sacs consist of fibrous thickening and infiltration of an exudate composed of fibrin, lymphocytes, mononuclear cells, and heterophils. Epithelial projections are present on the respiratory side of the air sac, where granulomatous foci also occur. Granulomas composed of immature reticular cells have been reported in the liver, myocardium, and kidneys.[9]

The lesions of ornithosis infection in the turkey included pneumonia, tracheitis, hepatitis, myocarditis, splenitis, orchitis, and fibrinous to fibrinopurulent inflammation of the air sacs and serous membranes.[2] While the pneumonia occurring in this infection was termed epithelioid pneumonitis, other changes in the respiratory system possessed many features in common with infectious sinusitis.

MATERIALS AND METHODS

The turkeys used in this project were from a PPLO- and ornithosis-free flock maintained on the Veterinary Research Farm of the Texas Agricultural Experiment Station. Turkeys in the flock were determined to be PPLO-free by the plate agglutination test, utilizing commercial antigen; the indirect complement-fixation test (ICF) was employed to establish the absence of ornithosis.

In the first study, 19 adult male turkeys, approximately 1 year old, were selected and again determined to be free of antibodies of both diseases. These birds were then allotted to four groups: three groups of 5 turkeys each and one group of 4 birds. Each group was placed in isolation in a security-type laboratory. Birds in group 1 were inoculated with T1 strain of PPLO,[6] those in group 2 with the Jo strain of the ornithosis agent,[5,14] and those in group 3 with a combination of the two agents. The remaining 4 birds were utilized as uninoculated controls.

The infectious agents had been propagated in chicken embryos obtained from hens which were serologically negative to the PPLO test. A mixture of infectious amnioallantoic fluid and yolk material was used for inoculation. Each turkey was given 0.25 ml. of the inoculum in the left maxillary[3] (paranasal, infra-orbital) sinus and a similar amount was given intratracheally. For birds in group 3, the inoculum was composed of equal amounts of the two agents.

Received for publication Jan. 23, 1960.

From the Department of Veterinary Pathology (Beasley) and the Department of Veterinary Microbiology (Moore, Watkins), Texas Agricultural Experiment Station, College Station.

This work was conducted in cooperation with and supported in part by funds made available by the Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture.

* Recently, this organism was classified as *Mycoplasma gallinarium* by Bergey's Manual of Determinative Bacteriology, 7th ed., 1957; however, the description differs in many aspects from the typical pathologic strain of PPLO used in this study.

TABLE 1—Serologic and Cultural Examination of Adult Turkeys Inoculated with PPLO, Ornithosis Agent, and Combination of Both Agents

	Postinoculation serology			Recovery of the agent	
	PPLO HI	PPLO Plate	Ornithosis ICF	PPLO	Ornithosis
CONTROL					
955	—	—	—	—	—
956	—	—	—	—	—
478	—	—	—	—	—
479	—	—	—	—	—
PPLO					
957	1/640	1/8	—	+	—
958	1/2,560	1/32	—	+	—
959	1/1,280	1/16	—	+	—
960	1/1,280	NS	—	+	—
961	1/1,280	1/16	—	+	—
ORNITHOSIS					
962	—	—	1/128	—	—
963	—	—	1/128	—	+
964	—	—	1/128	—	+
965	—	—	1/8	—	+
966	—	—	1/8	—	+
PPLO-ORNITHOSIS					
480		+*	1/128	+	+
481		+*	1/128	+	+
482		+*	1/256	—	+
483		+*	1/256	+	+
484		+*	1/256	+	+

— = negative; + = positive; NS = no sample; HI = hemagglutination inhibition; ICF = indirect complement fixation.

* Titer not determined.

TABLE 2—Serologic and Cultural Examination of 13-Week-Old Turkeys Inoculated with PPLO, Ornithosis Agent, and Combination of Both Agents

	Postinoculation serology		Recovery of the agent	
	PPLO Plate	Ornithosis ICF	PPLO	Ornithosis
CONTROL				
715	—	—	—	—
716	—	—	—	—
PPLO				
717	+*	—	—	—
718	+*	—	—	—
719	+*	—	+	—
720	+*	—	+	—
721	+*	—	+	—
ORNITHOSIS				
722	—	1/16	—	+
723	—	1/16	—	+
724	—	1/16	—	+
725	died	died	died	died
726	—	1/16	—	+
PPLO-ORNITHOSIS				
727	+*	1/16	+	+
728	+*	1/8	+	+
729	+*	1/8	—	+
730	+*	1/8	—	+
731	died	died	died	died

— = negative; + = positive; ICF = indirect complement fixation.

* Titer not determined.

Fourteen days after inoculation, all turkeys were exsanguinated. The gross lesions were recorded and exudates and tissues were obtained aseptically for bacterial and viral studies. Other tissues from the sinuses, trachea, lungs, air sacs, heart, spleen, liver, kidneys, pancreas, intestines, and testicles of each bird were fixed in 10 per cent formalin or Zenker's solution or both and embedded in paraffin. Tissue sections were cut at 6 μ and stained by Mayer's hemalum and eosin and Wolbach's modification of Giemsa's stain. Selected tissues were also stained by the Prussian blue, Gridley fungus, and Gridley reticulum methods.

Plate agglutination or hemagglutination inhibition tests for PPLO infection and ICF tests for ornithosis were performed on serum obtained from each bird just prior to necropsy. Sinus washings, air sac membranes, spleen, and testicles from each turkey were cultured in chicken embryos and on artificial medium in an attempt to recover the infectious agents. These procedures were carried out without the use of bacterial inhibiters.

In a second study, similar methods were employed with a group of 17 poults, 13 weeks old, which also were serologically negative to both PPLO and ornithosis. In this study, 2 birds were maintained as uninfected controls and the other groups consisted of 5 birds each.

RESULTS

The results of serologic and culture examinations are tabulated (tables 1, 2) and indicate that the control birds were not infected with either agent. There was no serologic evidence of mixed infection in the inoculated turkeys, except in those birds inoculated with both agents. Each turkey was negative to both diseases before inoculation and developed antibodies only against the organism or organisms with which it had been inoculated. Cultures did not reveal evidence of inadvertent or mixed infection, except in those birds inoculated with both agents.

Inoculated birds developed typical signs of the respective diseases. No mortality occurred in the adult birds and only 2 of the 13-week-old turkeys succumbed, 1 from ornithosis and the other from the mixed infection. A caseous exudate distended the inoculated sinuses of those turkeys given PPLO and a combination of PPLO and ornithosis agent, but did not occur in those birds inoculated with the ornithosis agent alone. Although the sinuses of some of the birds inoculated with the ornithosis agent did contain small amounts of mucus, most of the swelling occurred in the tissues of the face and subsided by the fourteenth day postinoculation. Otherwise, the signs

TABLE 3—Histopathologic Examination of Adult Turkeys Inoculated with PPLO, Ornithosis Agent, and Combination of Both Agents

	Sinusitis	Tracheitis	Pneumonia	Inflammation of the air sac	Pericarditis	Myocarditis	Hepatitis	Splenitis	Orchitis
CONTROL									
955	−	−	−	−	−	−	−	−	−
956	−	−	−	−	−	−	−	−	−
478	−	−	−	−	−	−	−	−	−
479	−	−	−	−	−	−	−	−	−
PPLO									
957	+	M	+	+	−	−	−	−	−
958	+	+	−	+	−	−	−	−	−
959	+	+	+	+	−	−	−	−	−
960	+	+	−	+	−	−	−	−	−
961	+	+	−	+	−	−	−	−	−
ORNITHOSIS									
962	+	+	−	+	+	+	+	−	+
963	+	+	−	+	+	+	+	+	+
964	+	+	+*	+	+	+	+	+	+
965	+	+	−	+	+	+	+	+	+
966	+	+	+	+	+	+	+	+	+
PPLO-ORNITHOSIS									
480	+	−	−	+	+	−	+	+	−
481	+	M	+	+	+	+	+	+	+
482	+	+	−	+	+	−	+	+	−
483	+	+	−	+	+	+	+	+	+
484	+	+	−	+	+	+	+	+	+

− = lesion not present; + = lesion present; M = missing.
* Aspergillosis.

and gross lesions were similar to those previously described.[4, 5, 7, 10-12, 21]

While lesions of divergent types were present in the parenchymatous organs of inoculated turkeys in the various groups, the lesions in the respiratory system were similar in turkeys of all three groups. The distribution of microscopic lesions is shown (tables 3, 4).

Ornithosis.—The lesions produced by the ornithosis agent were characterized by infiltration of mononuclear cells, combined with varying numbers of lymphocytes and heterophils. This type of infiltration oc-

TABLE 4—Histopathologic Examination of 13-Week-Old Turkeys Inoculated with PPLO, Ornithosis Agent, and Combination of Both Agents

	Sinusitis	Tracheitis	Pneumonia	Inflammation of the air sac	Pericarditis	Myocarditis	Hepatitis	Splenitis	Orchitis
CONTROL									
715	−	−	−	−	−	−	−	−	F
716	−	−	−	−	−	−	−	−	F
PPLO									
717	+	−	−	+	−	−	−	−	−
718	+	−	−	+	+	−	−	−	−
719	+	+	−	+	−	−	−	−	−
720	+	+	+	+	−	−	−	−	F
721	+	+	−	+	−	−	−	−	−
ORNITHOSIS									
722	+	+	+	+	+	−	−	+	F
723	+	+	−	+	+	+	+	+	−
724	+	+	−	+	−	−	+	+	−
725	+	+	+	+	+	−	+	−	−
726	+	+	−	+	+	+	+	+	−
PPLO-ORNITHOSIS									
727	+	+	+	+	+	−	+	−	F
728	+	+	+	+	+	−	+	−	F
729	+	+	+	+	+	−	+	−	−
730	+	+	+	+	+	−	+	+	F
731	+	−	+	+	+	−	−	−	−

− = lesion not present; + = lesion present; F = female.

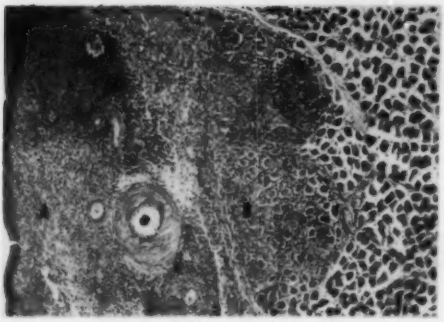


Fig. 1—Sinusitis in experimental ornithosis. Submucosal infiltration of lymphocytes and mononuclear cells (A) extending into the muscular layer of the sinus wall (B). H & E stain; x 55.

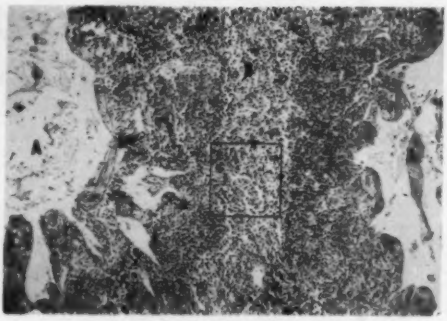


Fig. 2—Pneumonitis in ornithosis infection. Lumina of tertiary bronchi (A) relatively free of exudate. Interstitial tissue (B) heavily infiltrated by mononuclear cells and lymphocytes. H & E stain; x 65.

curred in the serous membranes, where the reaction was usually also fibrinous, and in the myocardium, liver, and testicles. In the liver, these infiltrations assumed scattered focal arrangements or were present in the portal canals; the sinusoids contained inflammatory cells of similar type. Kupffer's cells were engorged with massive amounts of an iron-positive, yellowish pigment, as were numerous macrophages in the spleen. Splenomegaly was produced by congestion, reticuloendothelial proliferation, and, in some, lymphoid proliferation. The necrotizing action of the agent was apparent only in the spleen of the poult dying from the infection and in the testes of the mature birds, where there was desquamation and necrosis of the germinal epithelium.

Lesions of varying intensity were produced in the respiratory system by the ornithosis agent. In the inoculated sinus, slight thickening of the mucosa was produced by infiltration of mononuclear cells, lymphocytes, and heterophils into the lamina propria. While the lymphocytes often were present in focal arrangements and occasionally occurred in the epithelium, the other inflammatory cells were diffusely spread through the connective tissue. Marked hypertrophic epithelial thickening did not occur. In contrast to the mild changes in the mucosa, there was marked infiltration of mononuclear cells and lymphocytes into the muscular layers of the sinus wall (fig. 1).

Tracheitis, characterized by cellular infiltration and deciliation, occurred in most of the inoculated turkeys. The mucosa was somewhat thickened by an infiltration of lymphocytes and mononuclear cells in the lamina propria. The cilia, normally present on the respiratory epithelium, were absent in the areas of most severe damage. Marked hypertrophy of the epithelium was not observed.

An epithelioid pneumonitis was produced in only 3 of the 10 turkeys inoculated with ornithosis. This lesion was characterized by infiltration of mononuclear cells and lymphocytes in the interstitial tissue between the tertiary bronchi (fig. 2, 3), the lumina of which contained an exudate consisting primarily of large epithelioid cells. While edema was present in the involved lungs, the exudation of fluid was not a salient feature of the inflammatory process. Lymphocytes, in focal and diffuse distribution, and heterophils accompanied the mononuclear cells but were not present in great numbers. Granulomatous nodules were often formed in the luminae of the tertiary bronchi.

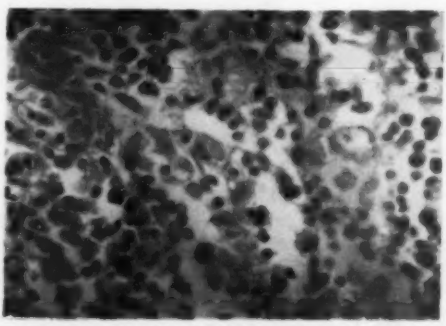


Fig. 3—Detail of interstitial infiltration indicated in figure 2. H & E stain; x 400.

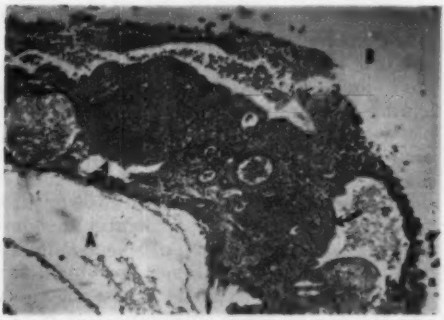


Fig. 4—Inflammation of air sac in ornithosis. Membrane thickened by infiltration of mononuclear cells and lymphocytes. Lymphocytic foci (arrows). Respiratory surface (B) covered by inflammatory exudate. Serosal surface (A). H & E stain; x 70.

Varying degrees of inflammation were present in the air sacs of each turkey in the ornithosis group. Thickening of these membranes was produced by edema and increased amounts of connective tissue with accompanying infiltrations of inflammatory cells in focal and diffuse distribution. The lymphocytes often occurred in focal or perivascular arrangements, while mononuclear cells and lymphocytes were distributed throughout the interstitial spaces (fig. 4). An exudate composed of fibrin, granulocytes, lymphocytes, and mononuclear cells, and often containing granulomatous foci, covered the respiratory side of the membrane. This exudate and the air sac were separated in some areas by a network of epithelial projections and spaces. While some of the spaces contained a few granulocytes, most were empty and were formed by hyperplastic epithelial cells, the active proliferation of which was confirmed by the presence of frequent mitotic figures.

PPLO Infection.—Except for increased numbers of lymphocytes, usually in organized follicles, the parenchymatous organs of the turkeys given only PPLO were without lesions. This increase of lymphoid tissue was observed in the liver, spleen, and kidneys of most turkeys, in the myocardium of 1, and in the gonads of 2 turkeys. Mild lymphocytic pericarditis occurred in 1 bird.

With the exception of the changes in the lungs, the lesions produced in the respiratory system by ornithosis and PPLO were similar. The inoculated sinus contained an exudate composed of fibrin, granulocytes, and cellular debris. Marked epithelial hypertrophy and infiltration of inflammatory cells thickened the mucosal lining of the sinus. There was no involvement of the deeper structures of the wall of the sinus.

The tracheitis produced by PPLO was similar to that of the ornithosis, with the addition of marked epithelial hypertrophy and the presence of occasional lymphocytic foci.

Pneumonia occurred in only 3 turkeys of the PPLO groups. In addition to increased numbers of lymphocytic foci, the histopathologic changes occurring in the lungs of these birds were characterized by diffuse exudation of albuminous fluid and inflammatory cells in the lumina of the bronchi and respiratory tubules (fig. 5, 6). This exudation in the bronchial tree and the diffuse infiltration, primarily of lymphocytes and granulocytes but also including mononuclear cells, partially obliterated the architecture in large areas of the lungs. Granulomatous foci were often present in

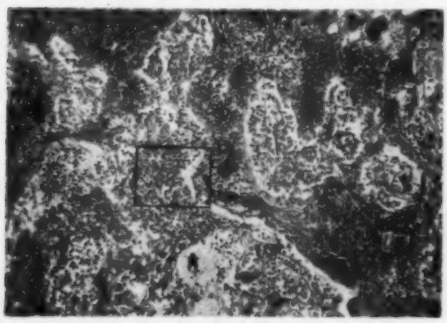


Fig. 5—Pneumonia in PPLO infection. Greatly dilated tertiary bronchus (A) containing numerous heterophils and abundant albuminous fluid. H & E stain; x 65.

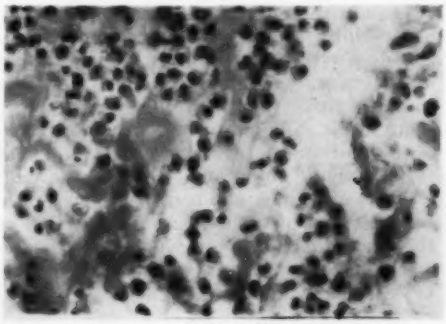


Fig. 6—Detail of inflammatory exudate indicated in figure 5. H & E stain; x 400.

the lumina of tertiary bronchi; in the more severely affected lungs, hypertrophy of the respiratory epithelium extended into the secondary and tertiary bronchi.

Lesions identical to those of ornithosis were present in the air sacs of all turkeys in the PPLO groups. These membranes were thickened; they contained focal and diffuse infiltrations of lymphocytes, heterophils, and mononuclear cells and were covered by a fibrinous exudate containing granulomatous nodules. Hyperplasia of the respiratory epithelium was also present.

Combination PPLO-Ornithosis.—The histopathologic changes in the turkeys inoculated with PPLO and the ornithosis agent consisted of the basic lesions of ornithosis with the addition of increased numbers of lymphocytic foci. However, the lesions in birds with mixed infection were more severe and extensive than those of turkeys with ornithosis alone. This fact was best represented in the lungs of the 13-week-old poults, in all 5 of which a severe epithelioid pneumonitis occurred. The necrotizing action of the ornithosis agent was evident only in the tissues of the poult dying from the infection.

Wolbach's modification of Giemsa's stain revealed the presence of minute elementary bodies only in the testes of 3 adult turkeys infected with ornithosis and in the tissues of the two 13-week-old poults dying from the infections. In the latter, these bodies occurred in cells of the inflammatory exudates and the reticuloendothelial cells of the spleen. Numerous cells containing clusters of elementary bodies occurred only in areas in which the necrotizing action of the agent was apparent. Elementary bodies were not observed in the tissues of the turkeys infected with PPLO alone.

DISCUSSION

The lesions observed in turkeys in this experiment were similar to those previously described as occurring in birds with PPLO [4,7,8,10,11,21] and ornithosis [2] infections. Histopathologically, the differentiating feature of these diseases is the septicemic nature of ornithosis and its affinity for the parenchymatous organs. Only increased numbers of lymphocytic foci occurred in the parenchymatous organs of the PPLO-infected turkeys; in the turkeys with ornithosis, myocarditis, hepatitis, splenitis, and orchitis were regularly observed. Inflammation of the testicles was a constant feature of ornithosis in the adult birds but did not occur in the 13-week-old turkeys.

While many similarities were noticed in the lesions produced in the respiratory system by these agents, certain differentiating features were present. Sinusitis has not been reported in field cases of virulent ornithosis. Thus, the lesions observed in the sinuses of the birds in the ornithosis groups may be considered an experimental phenomenon and, therefore, of only academic interest. Ornithosis did not produce the marked epithelial hypertrophy in the sinus and trachea or the lymphocytic foci in the tracheal mucosa that were present in birds infected with PPLO. Similar changes occurred in the air sacs of birds with both infections. Variations in the occurrence of these lesions probably represents degrees of severity in the reaction of the air sacs to an irritant. However, lymphocytic foci were often more numerous in birds with PPLO infections, and fibrin was more common in birds with ornithosis.

Disregarding the anatomical differences in the avian and mammalian lung and the special characteristics of avian inflammatory processes, the lesions produced in the lungs of turkeys by PPLO might be compared with those of a bacterial pneumonia in mammals.[1,20] The constituents of the inflammatory exudates and the manner of spread through the lung are comparable. However, the pulmonary lesions produced in turkeys by the ornithosis agent, characterized by interstitial infiltration and proliferation, and smaller amounts of fluid in an exudate composed principally of epithelioid cells might be compared to those of a viral pneumonitis in mammals.[1,20]

While the granulomatous foci occurring in these diseases bore a certain resemblance to the lesions of aspergillosis, mycelia were not demonstrated with Gridley fungus stains, with one exception. These foci might be considered attempts at resolution of the inflammatory exudates and, as such, an integral part of the infectious processes.[19] The 1 turkey in which fungus was present supports this supposition, since the granulomatous foci were scattered through the lungs in the absence of other changes.

The association of elementary bodies with the necrotizing action of the ornithosis agent is noteworthy. However, in ornithosis of other species in which necrosis is prominent, elementary bodies have not been regularly observed.[15-18]

SUMMARY AND CONCLUSIONS

The lesions produced by pleuropneumonia-like organisms (PPLO) and an ornithosis agent in 13-week-old and adult turkeys were studied histopathologically in both pure and mixed forms of infection. Serologic and culture methods were utilized to verify the type and purity of infection.

Except for an increased amount of lymphoid tissue in the parenchymatous organs, the lesions of PPLO were limited to the respiratory system. The ornithosis agent produced pericarditis, myocarditis, hepatitis, splenitis, and orchitis, the latter occurring only in sexually mature birds.

Lesions of the respiratory system, with the exception of minor variations, were similar in both diseases. Marked epithelial hypertrophy and lymphocytic foci were not observed in the tracheitis produced by ornithosis. Pneumonia with numerous lymphocytic foci occurred in those birds infected with PPLO, and epithelioid pneumonitis was present in the birds with ornithosis infections. Similar changes in the air sacs occurred in both diseases.

Combination PPLO-ornithosis infections produced more severe and extensive lesions which shared the characteristics of both diseases.

Elementary bodies were observed only in the testes of 3 adult turkeys infected with ornithosis and in the tissues of the 2 poults dying from ornithosis and PPLO-ornithosis infections.

References

[1] Anderson, W. A. D.: Pathology. 3rd ed. C. V. Mosby Co., St. Louis, (1957): 315, 660–669.

[2] Beasley, J. N., Davis, D. E., and Grumbles, L. C.: Preliminary Studies on the Histopathology of Experimental Ornithosis in Turkeys. Am. J. Vet. Res., 20, (March, 1959): 341–349.

[3] Chamberlain, F. W.: Atlas of Avian Anatomy. Hallenbeck Printing Co., Lansing, Mich., 1943.

[4] Cover, M. S., and Prier, J. E.: The Gross and Microscopic Pathology of Infectious Sinusitis of Turkeys. Cornell Vet., 39, (1949): 370–375.

[5] Davis, D. E., and Delaplane, J. P.: The Lesions of Ornithosis in Turkeys. In "Progress in Psittacosis Research and Control" (F. R. Beaudette, ed.), Rutgers Univ. Press, New Brunswick, N.J., 1958.

[6] Grumbles, L. C., Flowers, A. I., and Cassidy, D. R.: The Role of Pleuropneumonia-Like Organisms in Infectious Sinusitis of Turkeys. Avian Dis., 2, (1958): 336–347.

[7] Hitchner, S. B.: The Pathology of Infectious Sinusitis of Turkeys. Poult. Sci., 28, (1949): 106–118.

[8] Johnson, E. P.: The Specificity of Lymphofollicular Lesions in the Diagnosis of Chronic Respiratory Disease. Cornell Vet., 44, (1954): 230–239.

[9] Jungherr, E.: The Pathology of Experimental Sinusitis of Turkeys. Am. J. Vet. Res., 10, (Oct., 1949): 372–383.

[10] Jungherr, E.: The Pathology of Turkey Sinusitis. Proc. 20th Ann. Conf. Lab. Work. Pullorum Dis. Control, Mimeo., Virginia Dept. Agric., Richmond, 1948.

[11] Jungherr, E. L., Luginbuhl, R. E., and Jacobs, R. E.: Pathology and Serology of Air Sac Infection. Proc. Book, AVMA, (1953): 303–311.

[12] Jungherr, E. L., and Luginbuhl, R. E.: Air Sac Infection in Poultry. 56th Ann. Meeting U.S.L.S.A., 1952.

[13] Mataney, C. F., Pomeroy, B. S., and Osborn, O. H.: Studies on Egg Transmission of the Agent of Infectious Sinusitis of Turkeys. Proc. Book, AVMA, (1955): 310–315.

[14] Pate, D. D., Boney, W. A., Jr., and Delaplane, J. P.: Turkey Ornithosis. I. A Report of Natural and Experimental Infections. J.A.V.M.A., 125, (Dec., 1954): 476–478.

[15] Rivers, T. M., and Berry, G. P.: Psittacosis. II. Experimentally Induced Infection in Mice. J. Exptl. Med., 54, (1931): 105–118.

[16] Rivers, T. M., and Berry, G. P.: Psittacosis. III. Experimentally Induced Infection in Rabbits and Guinea Pigs. J. Exptl. Med., 54, (1931): 119–128.

[17] Rivers, T. M., and Berry, G. P.: Psittacosis. IV. Experimentally Induced Infections in Monkeys. J. Exptl. Med., 54, (1931): 129–144.

[18] Rivers, T. M., Berry, G. P., and Sprunt, D. H.: Psittacosis. I. Experimentally Induced Infection in Parrots. J. Exptl. Med., 54, (1931): 91–103.

[19] Runnells, R. A.: Animal Pathology. 5th ed. The Iowa State College Press, Ames, (1954): 142.

[20] Smith, H. A., and Jones, T. C.: Veterinary Pathology. Lea & Febiger, Philadelphia, 1957.

[21] Van Roekel, H., Gray, J. E., Shipkowitz, N. L., Clarke, M. K., and Luchini, R. M.: Chronic Respiratory Disease Complex in Chickens. University of Massachusetts Bull. No. 486, 1957.

SUMMARIO IN INTERLINGUA

Le Characteristicas Histopathologic de Morbos que Produce Inflammation in le Saccos de Aere de Gallos de India—Un Studio Comparative de Organismos Pleuropneumonioide e de Ornithosis in Infectiones Pur e Mixte

Le lesiones producite per organismos pleuropneumonioide (OPPO) e per un agente de ornithosis in gallos de India de 13 septimanas de etate e de etate adulte esseva studiate histopathologicamente in infectiones de forma pur e etiam mixte. Methodos serologic e culturatori esseva utilisate pro verificar le typo e le puritate del infectiones.

Con le exception de un accrescite quantitate de tissu lymphoide in le organos parenchymatose, le lesiones de OPPO esseva restringite al systema respiratori. Le agente de ornithosis

produceva pericarditis, myocarditis, hepatitis, splenitis, e orchitis. Iste ultime occurreva solmente in aves de sexualitate matur.

Le lesiones del systema respiratori—con le exception de minor variationes—esseva simile in le duo morbos. Marcate grados de hypertrophia epithelial e focos lymphocytic non esseva notate in le tracheitis producite per ornithosis. Pneumonia con numerose focos lymphocytic occurreva in le aves inficite per OPPO, e pneumonitis epithelioide esseva presente in le aves con infection per ornithosis. Simile alterationes in le saccos de aere occurreva in ambe morbos.

Infectiones combinate per OPPO e ornithosis produceva plus sever e plus extense lesiones que participava in le characteristicas de ambe morbos.

Corpores de inclusion esseva observate solmente in le testes de 3 adulte gallos de India inficite con ornithosis e in le tissu del 2 juvene aves que moriva de ornithosis e de infection per OPPO e ornithosis.

Newcastle Disease Immunity in Broilers and Caponettes Vaccinated with Killed-Virus and Live-Virus Vaccines

A. H. DARDIRI, D.V.M., Ph.D.; V. J. YATES, D.V.M.; P. W. CHANG, D.V.M.
D. E. FRY, B. A.

Kingston, Rhode Island

IN 1956, results of a study on three types of Newcastle disease (ND) vaccine for broilers and caponettes was reported.[4] The authors indicated that one dose of either the water or the dust type vaccine given to 5-day-old chickens resulted in poor protection; chickens succumbed to challenge. Revaccination with either type (water or dust) when chickens were 4 weeks old acted as a booster and produced 94 to 100 per cent immunity. Of chickens vaccinated with killed ND vaccine at 10 days of age, 100 per cent and 96.6 per cent were immune to challenge at 14 and 16 weeks of age, respectively.

Although reports by Waller *et al.*,[13] Schoening *et al.*,[11] Legenhausen *et al.*,[8,9] Hofstad,[5,6] and Sullivan *et al.*[12] substantiate that killed-virus ND vaccine produces satisfactory protection, the use of killed-virus vaccine has not gained popularity because of the method of administration and the early reports of low-grade protection resulting from the use of inactivated-virus vaccines.[1]

One of the main advantages of using killed-virus ND vaccine is the absence of vaccination reactions. Such reactions are usually associated with the administration of water, spray, and dust types of ND vaccines. Vigor and age of chickens, as well as management and environmental conditions, influence the severity of reaction. One advantage of the water and the dust type vaccines is that they can be administered to a large number of chickens without handling them individually; however, two vaccinations are necessary for satisfactory immunity.[4] In the field, general practice is to administer the first dose of water or dust vaccine sometime during the first week and the second dose at the end of the first month of age. Also, during its short lifetime a broiler or caponette may be subjected to stress situations such as debeaking, infectious bronchitis vaccination, hormonization, or unsuitable ventilation. These stress factors may result in a complicated respiratory condition termed "air sac."

The purpose of this report is to describe two experiments designed to (1) compare immunologic response to administration of killed vaccine alone, or followed by water or dust type vaccine and (2) test vaccination reaction following administration of killed- or live-virus ND vaccines in the presence of stress.

Materials and Methods

Experimental Birds.—Chickens used in this study were Rhode Island Reds obtained from the University of Rhode Island's Poultry Department. Their dams had been vaccinated with live-virus, wing-web ND vaccine at about 4 months of age. They were placed in an electric brooder at 1 day of age; after two weeks, they were allotted to groups, identified, and given specific treatments.

Facilities.—Throughout the study, birds were housed by group in separate pens. Chickens vaccinated with live-virus vaccines and those vaccinated with killed-virus vaccine were housed separately in an isolation facility 2 miles from the university farm. Good management was maintained during the experiment.

Vaccines, Newcastle Disease, and Infectious Bronchitis.—Three types of ND vaccines were used: water (Lasota strain), dust (B_1 strain), and killed-virus (California strain 11914, formalin-inactivated and aluminum hydroxide-adsorbed). They were purchased directly from the manufacturer. The infectious bronchitis (IB) vaccine (Massachusetts strain) was given intranasally.

Vaccination and Serology.—All ND vaccines were prepared and administered according to manufacturer's directions. Killed-virus vaccine was given intramuscularly. The first dose was 0.5 ml.; the second, 1.0 ml.

Blood samples were obtained from 15 chicks, 1 day old. The virus neutralization test was used to determine the prevaccination antibody titer.[3] Postvaccination blood samples were allowed to clot, and serum was collected and stored at −20 C. until tested. Antigenic response to vaccination was determined by the *beta* hemagglutination-inhibition

Received for publication Sept. 17, 1959.

Contribution 985 from the Rhode Island Agricultural Experiment Station, Kingston, R.I.

TABLE 1—Geometric Means of Hemagglutination-Inhibition Titers of Chicks Vaccinated with Killed-Virus or Live-Virus Newcastle Disease Vaccine, or Both

Group	Vaccine, week	Age in weeks										
		6	7	8	9	10	12	14	16	18	20	22
1	K2*	16**	45	54	76	64	27	38	14	16	14	10
2	K2K4	91	128	103	91	91	54	54	32	23	38	45
3	K2K6	....	64	76	91	91	91	91	54	54	38	38
4	K2W4	91	91	45	32	32	32	19	64	16	19	16
5	K2W6	....	128	107	128	76	64	45	23	54	45	32
6	W4	45	38	38	16	16	11	19	23	11	11	11
7	K2D4	128	128	76	64	76	54	45	38	32	32	32
8	K2D6	....	128	91	76	91	64	54	27	54	19	23
9	D4	64	76	64	27	19	27	32	45	7	14	10
10	Control	....	....	....	....	....	....	....	....	....	....	....

* K = killed-virus vaccine; D = dust vaccine; W = water vaccine; and No. = weeks of age at vaccination.
** Average geometric mean of the 4 birds selected at random from each group at the indicated week.

(HI) test.[4] Individual values obtained were converted to geometric means in accordance with the method of Markham.[10]

PPLO Testing.—The stained-antigen serum plate test was used to test for pleuropneumonia-like organism (PPLO) reactors. Blood was allowed to clot, and serum was separated. The latter was stored in a refrigerator at 4 C. for 24 hours before the test was conducted. The trachea of each bird was swabbed with sterile cotton attached to a wooden applicator stick. Specimens were immediately placed in 4 ml. of PPLO broth and frozen at −20 C. until tested. This test was carried out on each bird at 6 and 20 weeks of age. Three serial passages were made before a specimen was considered negative. The PPLO medium consisted of Difco PPLO broth without crystal violet, 1 per cent Difco serum fraction, 1/4,000 parts thallium acetate adjusted to pH 7.8, and 1,000 units of penicillin per milliliter.

Hormone Treatment.—At 6 and 9 weeks of age, birds were given 15 mg. of diethylstilbestrol (paste) subcutaneously in the neck region.

Challenge Exposure.—The challenge strain of ND virus used was Texas-GB-1948, propagated in 10-day embryonating chicken eggs. Upon the death of the embryos, the aminoallantoic fluids were harvested, tested for sterility, and pooled. Enough of the virus was prepared for all challenges; the pooled infectious material was distributed in 10-ml. vials and was stored at −20 C. until used. This highly virulent strain produced signs of paralysis or death, or both. The challenge inoculum was 0.25 ml. of virus dropped into the larynx of each bird, using a syringe. The titer of the fluid was 10^7 e.l.d.$_{50}$. The challenged birds were maintained under observation for two weeks in a wire battery, then were removed to an isolated pen where they were held for an additional six weeks. Interpretation of immunity was based on presence of paralysis, death, or both.

Challenged birds were cared for by a special attendant who had no contact with the other experimental chickens in the study.

PROCEDURE AND RESULTS

EXPERIMENT 1

Vaccine Combinations.—The 428 chickens used in the first experiment were allotted to ten groups (table 3). All birds in groups 1, 2, 3, 4, 5, 7, and 8 were vaccinated with killed-virus vaccine at 2 weeks of age. The chickens in groups 2 and 3 were given a second dose of killed-virus vaccine at 4 and 6 weeks of age, respectively. The birds in groups 4 and 5 were given water vaccine at 4 and 6 weeks of age, respectively; those in groups 7 and 8 were given dust vaccine at 4 and 6 weeks of age, respectively. One dose of water vaccine was given to the birds in group 6 when they were 4 weeks old; birds in group 9 were treated similarly with dust

TABLE 2—Results of Challenge in Birds Vaccinated with Live-Virus and Killed-Virus Newcastle Disease Vaccines

Group	Vaccine & week	Age in weeks when tested											No. resistant birds per total No.	Per cent resistant
		6	7	8	9	10	12	14	16	18	20	22		
1	K2*	4	4	4	4	4	4	4	2	4	4	2	40/44	91
2	K2K4	4	4	4	4	4	4	4	4	4	4	4	44/44	100
3	K2K6	0	4	4	4	4	4	4	4	4	4	4	40/40	100
4	K2W4	4	4	4	4	4	4	4	4	4	4	4	44/44	100
5	K2K6	0	4	4	4	4	4	4	4	4	4	4	40/40	100
6	W4	0	2	4	2	2	1	2	3	2	1	4	21/44	48
7	K2D4	4	4	4	4	4	4	4	4	4	4	4	44/44	100
8	K2D6	0	4	4	4	4	4	4	4	4	4	4	40/40	100
9	D4	4	4	2	2	2	2	2	3	2	2	3	30/40	68
10	Control	0	0	0	0	0	0	0	0	0	0	0	0/44	0

* K = killed-virus vaccine; D = dust vaccine; W = water vaccine; No. = weeks of age at time of vaccination.

vaccine. Birds in group 10 were left as unvaccinated controls.

Four chickens were taken from each of groups 1, 2, 4, 6, 7, 9, and 10 when birds were 6 weeks old. A blood sample for HI testing was collected from each of these birds before a challenge dose of ND virus was administered. This procedure was followed weekly through the tenth week, then every other week through the 22nd week. Chickens given a second dose of vaccine when 6 weeks old were treated similarly, starting at the seventh week.

Serologic Response to Vaccination.—The geometric means of the HI titers for vaccinated chickens are shown (table 1). Revaccination at 4 or 6 weeks of age resulted in an appreciable increase in HI levels as compared with the HI levels of chickens given single vaccinations. The HI antibody levels reached a peak between the seventh and tenth weeks, then diminished. At the end of the 22-week experimental period, birds vaccinated twice had maintained a higher HI level than birds given a single vaccination. A second vaccination, regardless of the type of vaccine, produced an anamnestic antibody response and increased the resistance against challenge.

A pool of serum samples collected when the birds were 1 day old neutralized 100 e.l.d.$_{50}$ of the Texas-GB-1948 ND virus.

Results of Challenge.—Birds chosen at random from each group were infected intratracheally at 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, and 22 weeks of age. Results of the challenge are shown (table 2). All nonvaccinated birds had typical respiratory and paralytic signs, followed by death within four days. Of the 44 birds given a single dose of killed-virus vaccine when 2 weeks old (group 1), 4 birds died, 2 after challenge at the sixteenth week and 2 after challenge at the 22nd week. Ninety-one per cent of group 1 birds were resistant to challenge. Of the 44 birds given a single dose of water vaccine when 4 weeks old (group 6), 21 were not protected against challenge. Mortality was distributed between the seventh and 22nd weeks. Of the group 6 birds, 48 per cent were resistant to challenge. Of the group 9 birds, given a single dose of dust vaccine at 4 weeks of age, 30 (68%) were resistant to challenge.

All birds given two vaccinations were resistant to intratracheal challenge during the 22 weeks of the experiment.

EXPERIMENT 2

Vaccination in the Presence of Stress.—In the second experiment, 460 chickens were used to study the reaction to vaccination in the presence of stress. In the parent flock, 50 per cent of the birds were tested for PPLO antibodies, and more than half of them were positive. At the time of hatching, chicks were identified and brooded in an electric battery. At the end of two weeks, they were allotted at random to five groups. Groups 1, 2, and 3 consisted of 80 birds each; there were 110 birds in group 4 and 110 in group 5. The number of birds in each group was adjusted so that all birds were provided with approximately the same floor area. All birds were vaccinated intranasally with IB vaccine at 2 weeks of age. At the same time, birds in groups 2 and 3 were vaccinated with killed-virus ND vaccine. Those in groups 4 and 5 were given the water type of live-virus ND vaccine; they were kept on a farm 2 miles away from birds in the other three groups. The chickens in group 1 served as controls; they were not given ND vaccine and were housed separately from those in groups 2 and 3.

When the chickens were 6 weeks old, they were revaccinated with ND vaccine (killed-virus vaccine for those that had it before, and dust vaccine for those previously vaccinated with the water type). At this time, groups were subdivided by randomly allot-

TABLE 3—Plan of Experiment on Vaccination Reactions Following Administration of Newcastle Disease Vaccine in the Presence of Stress

Treatment or test	Group No.									
	1A	1B	2A	2B	3A	3B	4A	4B	5A	5B
2 WEEKS										
IB vaccine	+	+	+	+	+	+	+	–	+	+
Killed-virus ND vaccine	...	...	+	+	+	+	...	...	...	...
Water type ND vaccine	...	...	...	...	...	...	+	–	+	+
6 WEEKS										
PPLO serum test	+	+	+	+	+	+	–	–	+	+
Killed-virus ND vaccine	...	...	+	+	+	+	...	...	...	...
Dust type ND vaccine	...	...	...	...	...	...	+	–	+	+
Diethylstilbestrol	+	...	+	...	+	...	+	...	+	...
9 WEEKS										
Diethylstilbestrol	+	...	+	...	+	...	+	...	+	...
20 WEEKS										
PPLO culture	+	+	+	+	+	+	+	+	+	+

Plus signs indicate that birds were given the designated treatment or test.

ing the birds in each group to two pens. Birds in subgroups 1A, 2A, 3A, 4A, and 5A were given diethylstilbestrol. The hormone treatment was repeated when birds were 9 weeks old. At 6 weeks of age, 50 per cent of the chickens in each subgroup were selected at random and blood samples were taken for PPLO testing. At the end of the experiment, when birds were 20 weeks old, 50 per cent of them were tested for PPLO by culturing tracheal swabs. The design of the experiment is shown (table 3).

Vaccination Reactions.—Birds in groups 4 and 5, given water type ND vaccine and intranasal IB virus, were affected by sneezing during the two weeks following vaccination. About 10 per cent of the birds manifested wet eyes and nasal discharge; some had swollen sinuses. Some became weak and depressed. Approximately 2 per cent of the birds died within two weeks after vaccination. Inflammation of the tracheal lining was found upon postmortem examination. In some of these birds, yellow caseous plugs were seen in the lower part of the trachea and in the bronchi. All of the dead birds had various degrees of air sac involvement. About 50 per cent of them had yellow, creamy, or caseous material in their air sacs and almost 25 per cent had livers covered with a fibrinous exudate. The virus of ND was isolated from the tracheal discharge. This material was also cultured for PPLO. Exudate from the air sacs and pericardiums was cultured for PPLO and bacteria. Only *Escherichia coli* was isolated from the air sacs and pericardiums. There was no evidence of PPLO infection upon serum testing at six weeks and culturing of tracheal swabs at 22 weeks.

The birds given killed-virus vaccine and IB virus and the control birds had signs of mild respiratory involvement. These birds recovered and signs were not evident one week after vaccination.

DISCUSSION

The first experiment in this study determined the degree and duration of immunity following the administration of killed-virus ND vaccine at 2, 2 and 4, or 2 and 6 weeks of age and killed-virus ND vaccine at 2 weeks of age followed by a dose of live-virus ND vaccine in water or as dust two or four weeks later.

In general, birds given a single dose of vaccine at 2 weeks of age had a lower degree of immunity than did those given a second dose of vaccine. As a result of one dose of water or dust vaccine, 48 and 68 per cent, respectively, were resistant to challenge. Of the birds given one dose of killed-virus vaccine, 91 per cent were immune (table 2). All the birds that were vaccinated twice were resistant when challenged. These results agree with the work of Dardiri *et al.*,[4] who showed that a second dose of vaccine was necessary if chickens were to be kept longer than 12 weeks of age.

Chickens that were given a second dose of vaccine were shown to have 100 per cent resistance when their immunity was challenged. They also had a heightened serologic response following the second dose whether it was killed-virus or live-virus vaccine or given at two or four weeks after first vaccination. A postvaccination reaction to the second dose of vaccine was never observed.

Similar observations regarding anamnestic response to a second injection of inactivated ND vaccine have been reported by Brandly *et al.*,[2] Waller *et al.*,[13] and Hofstad,[5,6] who emphasized the importance of the time interval between injections in establishing the optimum response. Serologic evidence of a heightened state of immunity and resistance to challenge following a booster injection with killed-virus vaccine was demonstrated by Lægenhausen.[8,9]

Winterfield and Seadle[14] stated that when 4½-week-old chickens were vaccinated through the drinking water or by the ocular route, serologic tests performed 12 weeks after vaccination indicated a need for revaccination before sexual maturity.

Successful protection of layers by a single dose of killed-virus vaccine at 10 days or 2 weeks of age and revaccination at 3½ months of age with a live-virus vaccine was reported by Koch.[7]

The occurrence of complete protection in all given a booster injection of vaccine two or four weeks after the primary inoculation is noteworthy. Four weeks was the longest interval employed in this study. The optimum interval between the time of first and second injections of vaccine may be beyond this point, as indicated by Hofstad.[5,6]

In the second experiment, birds vaccinated with live-virus ND vaccine in water and with IB intranasally had a severe respiratory reaction. Newcastle disease virus was isolated from the 2 per cent that died

and all of these birds had lesions of the "air sac" syndrome. These birds recovered completely from the respiratory reaction, but growth was uneven. None of the birds in the control group were clinically affected.

Although many of the parents of these chickens were PPLO reactors, it was not possible to isolate PPLO from the dead birds. Only *Esch. coli* was isolated from the air sacs and pericardial exudates. At six weeks, the serum from 50 per cent of these birds was negative for PPLO antibodies. At 22 weeks, PPLO was not isolated from the tracheas of the 50 per cent tested. This is hard to explain, as these chickens were "stressed" by hormone treatment at 6 and 9 weeks of age. However, the authors obtained similar results in another experiment investigating the stress factors that may influence the appearance of air sac lesions.

SUMMARY

This report concerns the immunity and vaccination reaction following the use of a single dose of killed- or live-virus Newcastle disease vaccine and revaccination with a second dose of killed- or live-virus vaccine two or four weeks after the first dose.

Results of one experiment indicated that a single dose of vaccine, killed-virus or live-virus, did not provide adequate protection against intratracheal challenge with the Texas-GB-1948 strain of Newcastle disease virus. Revaccination with killed- or live-virus vaccine produced an anamnestic response, and 100 per cent of the birds were protected against intratracheal challenge at 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, and 22 weeks of age.

From further experimental trials, it was found that lesions of "air sac" syndrome resulted when birds were given live Newcastle disease and infectious bronchitis vaccine when 2 weeks old. Serologic and isolation tests for pleuropneumonia-like organisms were negative when conducted at six and twenty weeks, respectively.

References

[1] Beaudette, F. R.: Twenty Years of Progress in Immunization Against Virus Diseases of Birds. J.A.V.M.A., 115, (Oct., 1949): 232–244.

[2] Brandly, C. A., Moses, H. E., Jones, E. E., and Jungherr, E. L.: Immunization of Chickens Against Newcastle Disease. Am. J. Vet. Res., 7, (July, 1946): 307–332.

[3] Cunningham, C. H.: A Laboratory Guide in Virology. Burgess Publishing Co., Minneapolis, 1956.

[4] Dardiri, A. H., Chang, P. W., and Fry, D. E.: Immunity Study of Three Types of Newcastle Disease Vaccine for Broilers and Caponettes. Am. J. Vet. Res., 18, (April, 1957): 400–404.

[5] Hofstad, M. S.: Immunization of Chickens Against Newcastle Disease by Formalin-Inactivated Vaccine. Am. J. Vet. Res., 14, (Oct., 1953): 586–589.

[6] Hofstad, M. S.: The Secondary Immune Response in Chickens Revaccinated with Inactivated Newcastle Disease Virus Vaccine. Am. J. Vet. Res., 15, (Oct., 1954): 604–606.

[7] Koch, B.: Symposium on Newcastle Disease Vaccines. Proc. Book, AVMA, (1955): 332.

[8] Leganhausen, D. H., and Sinkiewicz, R. J.: Studies of Newcastle Disease. II. Evaluation of Two Killed Newcastle Disease Vaccines. Avian Dis., 3, (1959): 3–11.

[9] Leganhausen, D. H., Sinkiewicz, R. J., and Sullivan, J. F.: Studies of Newcastle Disease. III. Further Studies of a Killed Newcastle Disease Vaccine. Avian Dis., 3, (1959): 12–22.

[10] Markham, F.: The Geometric Mean Titer in Serologic Studies. Technical Information. American Cyanamid Co., Pearl River, N.Y., (1955): 1–5.

[11] Schoening, H. W., Osteen, O. L., Leganhausen, D. H., Anderson, W. A., and Hall, W. J.: Vaccination Against Newcastle Disease with Formalin-Inactivated, Commercially Produced Vaccines. Am. J. Vet. Res., 10, (April, 1949): 176–182.

[12] Sullivan, J. F., Gill, E., and Somer, A. M.: Immune Response of Chickens to *Beta*-Propiolactone-Killed Newcastle Disease Vaccines. Am. J. Vet. Res., 19, (April, 1958): 483–488.

[13] Waller, E. F., and Gardiner, M. R.: Newcastle Disease: Response to Formalized Vaccine. Poult. Sci., 32, (1953): 405–411.

[14] Winterfield, R. W., and Seadle, E. H.: Newcastle Disease Immunization Studies. II. The Immune Response of Chickens Vaccinated with Newcastle Disease Virus Administered Through the Drinking Water. Poult. Sci., 36, (1957): 54–64.

SUMMARIO IN INTERLINGUA

Immunitate a Morbo de Newcastle in Juvene Gallinas e Capones Vaccinate con Vaccinos a Virus Morte e a Virus Vive

Iste reporto concerne le immunitate e le reaction vaccinational post le uso de un sol dose de vaccino de morbo de Newcastle a virus morte o a virus vive, sequite post un intervallo de duo o quatro septimanas per un revaccination con un secunde dose de vaccino a virus morte o a virus vive.

Le resultatos de un experimento indicava que un sol dose de vaccino a virus morte o a virus vive non provide un adequate protection contra le provocation intratracheal con le racia

Texas-GB-1948 de virus de morbo de Newcastle. Revaccination con vaccino a virus morte o a virus vive produceva un reaction anamnestic, e 100 pro cento del aves esseva protegite contra le provocation intratracheal al etates de 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, e 22 septimanas.

In altere experimentos il esseva trovate que lesiones del syndrome de "sacco de aere" resultava quando le aves recipeva al etate de 2 septimanas vaccino vive de morbo de Newcastle e bronchitis infectiose. Tests serologic e tests de isolation pro organismos pleuropneumonioide esseva negative quando illos esseva executate al etates de sex e vinti septimanas respectivemente.

Enzyme Activity Levels in Chicken Plasma

L. S. McDANIEL, D.V.M., M.S., and H. L. CHUTE, D.V.M., D.V.Sc.

Orono, Maine

NUMEROUS reports have been published concerning the activity levels of glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), lactic dehydrogenase (LDH), malic dehydrogenase (MDH), acid phosphatase (acidPH), and alkaline phosphatase (alkPH) in human and mammalian blood. There is little information, however, relative to the levels of these enzymes in chicken blood. The majority of this information is concerned with alkPH levels.[1-3, 7, 12, 14-22, 34] Plasma acidPH levels of adult, single-comb White Leghorns (WL) have also been measured.[14] The GOT levels in whole blood of WL chicks, serum of adult WL, and plasma of New Hampshire Red chickens have been reported.[9-11] The MDH activity of avian erythrocytes was investigated by Rubenstein and Danstedt.[25]

Many procedures for measuring enzymatic activities have been described in the literature. The objectives of this study were to investigate various assay procedures in order to determine their applicabilities for avian plasma and to measure enzyme activity levels of plasma from normal chickens.

MATERIALS AND METHODS

The experimental birds were WL and White Plymouth Rocks (WR) which were hatched at the Department of Animal Pathology, University of Maine, and housed in isolation units. Heparinized blood samples were collected by cardiac puncture. Hemolyzed plasma specimens were discarded. In most instances, the plasma was stored overnight at −5 C. Duplicate enzyme determinations were conducted whenever the quantities of the available materials permitted.

The assay methods and commercially available substrates employed to measure GOT, GPT, LDH, and MDH activity levels are listed (table 1). The principle of the colorimetric procedure used for measuring acidPH and alkPH activities[27] is similar to the one described by Bessey *et al.*[5] The plasma samples were diluted 1:10 with distilled water for the alkPH assay and the GOT assay by the colorimetric procedure.

The optical instruments employed included a Klett-Summerson photoelectric colorimeter, a Beckman DU spectrophotometer, and a Coenzometer.* The latter instrument, described by Thiers and Vallee,[32] has a single wavelength of 350 mμ. Assays on the spectrophotometer were conducted at 340 mμ.

RESULTS

The various analytical procedures for the enzymes were compared by computation of the correlation coefficients.[29] A summary of the statistical analyses is presented (table 1). The accuracy of the colorimetric procedures for GOT, GPT, and LDH was not affected by the optical instrument employed. According to the paired Student's "t" test,[29] there were no significant statistical differences in the values obtained by means of the colorimeter and the spectrophotometer at 505 mμ. Plasma samples from 20 seven-week-old WL were tested.

Enzyme activity levels detected in plasma from normal chickens are given (table 2–4). The unpaired Student's "t" test[29] was used to analyze the differences in the plasma enzyme levels between breeds and sexes. Plasma samples from 15 six-month-old roosters and 15 laying hens of the same age were tested for GOT, GPT, LDH, and MDH by all the spectrophotometric procedures. The average enzyme activity level detected by each method was lower in the hens than the roosters, but the differences were not significant. The breed comparisons were conducted on two groups of birds in the following manner: the GOT, MDH, and LDH activities in plasma from 18 six-month-old WL and 12 six-month-old WR were measured by spectrophotometric procedures; GOT, GPT, and acidPH activities in plasma from 17 eight-week-old WL and 10 eight-week-old WR were measured by colorimetric proce-

Received for publication Jan. 29, 1960.

From the Department of Animal Pathology, University of Maine, Orono.

This project was supported in part by the Fort Halifax Packing Co., Winslow, Maine, and in part by research grant C-4957 (C1) from the National Institutes of Health, U.S. Public Health Service.

The authors thank Warner Chilcott Co., Morris Plains, N.J., for supplying part of the transamination substrates.

* Manufactured by Macalaster Bicknell Co., Cambridge, Mass.

TABLE 1—Correlation of Methods for Measurement of Plasma Enzyme Activities in Chickens

Method	Assay methods: Commercial substrates*	Assay methods: Instrument	Methods compared	No. birds	Correlation coefficient
GLUTAMIC OXALACETIC TRANSAMINASE					
(A) Karmen.[13]	Warner-Chilcott	Spectrophotometer	A & B	71	0.043**
(B) Karmen.[13]	Worthington	Coenzometer	A & C	16	0.404**
(C) Reitman and Frankel [24]; Sigma Tech. Bull. 505.[25]	Sigma	Colorimeter	B & C	13	0.094**
GLUTAMIC PYRUVIC TRANSAMINASE					
(D) Wroblewski and LaDue.[35]	Warner-Chilcott	Spectrophotometer	D & E	15	0.343**
(E) Reitman and Frankel [24]; Sigma Tech. Bull. 505.[25]	Sigma	Colorimeter			
LACTIC DEHYDROGENASE					
(F) Wroblewski and LaDue.[35]		Spectrophotometer	F & G	59	0.497†
			F & H	72	0.628†
(G) Wroblewski and LaDue.[35]		Coenzometer	F & I	44	0.631†
			G & H	59	0.521†
(H) Wacker *et al.*[32]	Worthington	Coenzometer	G & I	29	0.327**
			H & I	41	0.016**
(I) Berger and Broida [4]; Cabaud and Wroblewski.[6]	Sigma	Colorimeter			
MALIC DEHYDROGENASE					
(J) Siegel and Bing.[26]		Spectrophotometer	J & K	46	0.189**
(K) Siegel and Bing.[26]		Coenzometer			

* Warner-Chilcott Co., Morris Plains, N. J.; Worthington Biochemical Corp., Freehold, N. J.; Sigma Chemical Co., St. Louis, Mo.

** Not significant.

† Significant at the 1 per cent level.

dures. No significant statistical differences were found.

Discussion

Many factors, including pH, temperature, quantity of material assayed, quantities of reagents used, and various ions may affect enzymatic activities. [23] The analytical procedures measure the level of the activity of an enzyme under specific conditions rather than the actual concentration of an enzyme, although the level may vary according to the amount of the enzyme present. Therefore, values obtained by various methods are not necessarily comparable. The lack of correlation between the different methods employed in this study, except for three of the LDH assay methods, emphasizes this fact. The standard deviations listed (tables 2–4) indicate that there is considerable biologic variation and that the width of the ranges in normal birds is dependent upon the assay method employed. A relatively small difference in plasma enzyme levels according to one method may be more significant than a relatively large difference according to another method. At the present time, no absolute values can be given for enzyme activity levels in avian blood. All experimental data must be analyzed on a relative basis. Statistics are a useful tool in this respect.

Qualities which were desired in the assay methods included a rapid procedure with a minimum of manipulation, relatively stable reagents, and reproducible results. Among the methods attempted, the following are preferred: LDH activity by the method of Wroblewski and LaDue [35] at 340 mμ, GOT activity by the method of Karmen [13] at 340 mμ, and GPT activity by the method of Reitman and Frankel.[24] The method of measuring MDH activity which was used in this study is not considered entirely satisfactory. The methods employed to measure acidPH and alkPH activity levels appear to be suitable for avian plasma. The sensitivity of the alkPH assay, however, is de-

creased by the necessary dilution of the plasma.

None of the plasma enzyme levels observed in these trials, except the alkPH values, could be compared with data in the literature. Previously reported values were not measured by the same procedures. Cornelius *et al.*[9] did not detect any GPT activity in serum from adult WL by a colorimetric method. No activity could be demonstrated in some of the plasma samples during this investigation, but a majority of the samples contained a low level of GPT activity.

The alkPH levels reported here are similar to the values reported by Tanabe and Wilcox,[31] lower than the values reported by Bell,[2] and higher than the values reported by Stutts *et al.*[30] The same substrate, p-nitrophenyl phosphate, was utilized in all of these studies. However, a different buffer at a slightly higher pH was used in this investigation. Several authors have reported that alkPH levels were lower in growing chickens than in adult birds.[8, 14, 31] Similar observations were made during this study.

Age does not appear appreciably to affect the ranges of plasma GOT, GPT, and acidPH levels in chickens between the ages of 5 weeks and 6 months. The average LDH level of 6-month-old chickens is lower than

TABLE 2—Transaminase Activity Levels in Chicken Plasma

Assay method	Age of birds	No. birds tested	Units/ml. plasma (mean ± s.d.)
GLUTAMIC OXALACETIC TRANSAMINASE			
Method of Karmen* at 340 mμ	5 wk.	30	160 ± 13
	6 wk.	16	187 ± 23
	7 wk.	42	134 ± 38
	8 wk.	10	156 ± 22
	10 wk.	30	154 ± 27
	14 wk.	30	180 ± 39
	6 mo.	30	171 ± 59
Method of Karmen* at 350 mμ	6 wk.	13	144 ± 28
	7 wk.	16	155 ± 31
	8 wk.	10	111 ± 31
	6 mo.	30	136 ± 38
Method of Reitman and Frankel**	5 wk.	10	196 ± 84
	6 wk.	21	202 ± 105
	7 wk.	28	260 ± 81
	8 wk.	27	179 ± 71
	10 wk.	20	214 ± 58
	16 wk.	20	214 ± 85
	6 mo.	30	177 ± 86
GLUTAMIC PYRUVIC TRANSAMINASE			
Method of Wroblewski and LaDue* at 340 mμ	5 wk.	26	5 ± 7
	6 wk.	16	11 ± 10
	8 wk.	10	5 ± 2
	6 mo.	30	8 ± 6
Method of Reitman and Frankel**	5 wk.	10	16 ± 8
	6 wk.	20	4 ± 9
	7 wk.	25	16 ± 8
	8 wk.	27	13 ± 4
	10 wk.	20	14 ± 7
	16 wk.	20	14 ± 6
	6 mo.	30	13 ± 7

* One unit = amount of enzyme activity which will cause a decrease of 0.001 in optical density per minute under standard conditions.

** Activity expressed in *Sigma* Frankel units.

TABLE 3—Dehydrogenase Activity Levels in Chicken Plasma

Assay method	Age of birds	No. birds tested	Units/ml. plasma (mean ± s.d.)
LACTIC DEHYDROGENASE			
Method of Wroblewski and LaDue* at 340 mμ	5 wk.	30	844 ± 113
	6 wk.	15	944 ± 229
	7 wk.	46	736 ± 242
	10 wk.	30	918 ± 158
	14 wk.	30	870 ± 165
	6 mo.	30	447 ± 171
Method of Wroblewski and LaDue* at 350 mμ	7 wk.	18	554 ± 215
	6 mo.	30	200 ± 120
Method of Wacker *et al.***	6 wk.	12	262 ± 58
	7 wk.	16	224 ± 89
	8 wk.	10	244 ± 88
	6 mo.	30	143 ± 54
Method of Berger and Broida†	5 wk.	10	801 ± 124
	6 wk.	20	817 ± 358
	7 wk.	16	600 ± 300
	8 wk.	27	490 ± 288
	10 wk.	20	418 ± 252
	16 wk.	16	493 ± 153
	6 mo.	30	615 ± 307
MALIC DEHYDROGENASE			
Method of Siegel and Bing* at 340 mμ	5 wk.	30	107 ± 14
	6 wk.	16	160 ± 63
	7 wk.	46	103 ± 52
	8 wk.	17	143 ± 42
	10 wk.	30	121 ± 41
	14 wk.	30	50 ± 39
	6 mo.	30	81 ± 27
Method of Siegel and Bing at 350 mμ	7 wk.	9	40 ± 21
	6 mo.	23	28 ± 12

* One unit = amount of enzyme activity which will cause a decrease of 0.001 in optical density per minute under standard conditions.

** One unit = amount of enzyme activity which will cause increase of 0.001 in optical density per minute under standard conditions.

† Activity expressed in Berger-Broida LDH units.

TABLE 4—Phosphatase Activity Levels in Chicken Plasma

Age of birds	No. birds tested	Acid phosphatase* (mean ± s.d.)	No. birds tested	Acid phosphatase* (mean ± s.d.)
5 wk.	40	0.325 ± 0.205	41	46 ± 17
6 wk.	17	0.315 ± 0.152	17	25 ± 15
7 wk.	15	0.408 ± 0.196	15	18 ± 7
8 wk.	27	0.682 ± 0.239	17	19 ± 14
10 wk.	20	0.495 ± 0.257	20	30 ± 8
6 mo.	30	0.480 ± 0.277	30	8 ± 5

* Enzyme activity expressed in Bessey-Lowry (B-L) units. One B-L unit (Sigma) equals the quantity of phosphatase which will liberate one mM of p-nitrophenol per hour per liter of serum.

the average levels of the younger birds. The data obtained by the colorimetric method disagree with this observation, but this method is considered the least reliable of the methods used for chicken plasma. The average MDH levels of the 14-week-old and the 6-month-old chickens were also somewhat lower than the average levels of birds tested at an earlier age.

Summary

The applicabilities of various enzyme assay procedures for avian plasma were investigated. Modifications of the following procedures are suitable: the method of Wroblewski and LaDue for lactic dehydrogenase (LDH), method of Siegel and Bing for malic dehydrogenase (MDH), the method of Karmen for glutamic oxalacetic transaminase (GOT), the method of Reitman and Frankel for glutamic pyruvic transaminase (GPT), and the method of Bessey *et al.* for acid phosphatase (acidPH) and alkaline phosphatase (alkPH).

The levels of the enzymes were measured in plasma from normal, single-comb White Leghorns and White Plymouth Rocks, 5 weeks to 6 months in age. The average GOT, GPT, and acidPH levels of chickens in the various age groups were similar. The average LDH, MDH, and alkPH levels were lower in adults than in younger birds. No significant statistical differences were observed between the average plasma GOT, GPT, LDH, and MDH levels of males and females or the average plasma GOT, GPT, LDH, MDH, and acidPH levels of the two breeds.

Addendum

Since this article was written, the MDH assay for chicken plasma has been improved. Reproducible values may be obtained by the following modification of the method of Siegel and Bing.[26] A mixture containing 2.7 ml. of 0.1 M phosphate buffer (pH 7.5), 0.1 ml. of reduced diphosphopyridine nucleotide (5.0 mg./ml.), and 0.1 ml. of plasma is incubated for 30 minutes at 32 C. The reaction is then initiated by 0.1 ml. of 0.1 M oxalacetic acid. The optical density of the reaction at 340 mμ is recorded at intervals of 30 seconds for two minutes.

References

[1] Auchinachie, D. W., and Emslie, A. R. G.: The Significance of Phosphatase Estimations in the Adult Fowl. Biochem. J., 28, (1934): 1993–2001.

[2] Bell, D. J.: Tissue Components of the Domestic Fowl. 4. Plasma Alkaline Phosphatase Activity. Biochem. J., 75, (1960): 224–229.

[3] Bell, D. J., Siller, W. G., and Campbell, J. G.: Observations on "Cage Layer Fatigue" (CLF) in Hens. Biochem. J., 72, (1959): 32P.

[4] Berger, L., and Brioda, D.: The Colorimetric Determination of Lactic Dehydrogenase in Serum or Other Fluids at 400 to 500 mμ. Tech. Bull. 500. Sigma Chemical Co., St. Louis, Mo., 1959.

[5] Bessey, O. A., Lowry, O. H., and Brock, M. J.: A Method for the Rapid Determination of Alkaline Phosphatase with Five Cubic Millimeters of Serum. J. Biol. Chem., 164, (1946): 321–329.

[6] Cabaud, P. G., and Wroblewski, F.: Colorimetric Measurement of Lactic Dehydrogenase Activity of Body Fluids. Am. J. Clin. Path., 30, (1958): 234–236.

[7] Common, R. H.: Serum Phosphatase in the Domestic Fowl. Nature, 133, (1934): 572.

[8] Common, R. H.: Serum Phosphatase in the Domestic Fowl. J. Agric. Sci., 26, (1936): 492–508.

[9] Cornelius, C. E., Bishop, J., Switzer, J., and Rhode, E. A.: Serum and Tissue Transaminase Activities in Domestic Animals. Cornell Vet., 49, (1959): 116–126.

[10] Cornelius, C. E., Law, G. R. J., Julian, L. M., and Asmundson, V. S.: Plasma Aldolase and Glutamic Oxalacetic Transaminase Activities in Inherited Muscular Dystrophy of Domestic Chicken. Proc. Soc. Exptl. Biol. & Med., 101, (1959): 41–44.

[11] Goswami, M. N. D., and Robblee, A. R.: Aspartic-Glutamic Transaminase Activity in Chick Liver. Poult. Sci., 37, (1958): 96–99.

[12] Gutowska, M. S., Parkhurst, R. T., Parrott, E. M., and Verburg, R. M.: Alkaline Phosphatase and Egg Formation. Poult. Sci., 22, (1943): 195–204.

[13] Karmen, A.: A Note on the Spectrophotometric Assay of Glutamic Oxalacetic Transaminase in Human Blood Serum. J. Clin. Invest., 34, (1955): 131–133.

[14] Lesher, S., and Burmester, B. R.: Plasma Phosphatase Activities of Normal and Lymphomatous Chickens. Cancer Res., 15, (1955): 537–540.

[15] Motzok, I.: Studies on the Plasma Phosphatase of Normal and Rachitic Chicks. 2. Relationship Between Plasma Phosphatase and the Phosphatase of Bone, Kidney, Liver, and Intestinal Mucosa. Biochem. J., 47, (1950): 193–196.

[16] Motzok, I.: Studies on the Plasma Phosphatase of Normal and Rachitic Chicks. 3. The Assay of Antirachitic Preparations by a Method Based on the Determination of Plasma Phosphatase Activity. Biochem. J., 47, (1950): 196–199.

[17] Motzok, I.: Studies on Alkaline Phosphatases. 1. Kinetics of Plasma Phosphatase of Normal and Rachitic Chicks. Biochem. J., 72, (1959): 169–177.

[18] Motzok, I., and Branion, H. D.: Influence of Fluorine on Phosphatase Activities of Plasma and Tissues of Chicks. Poult. Sci., 37, (1958): 1469–1471.

[19] Motzok, I., and Branion, H. D.: Studies on Alkaline Phosphatases. 2. Factors Influencing pH Optima and Michaelis Constant. Biochem. J., 72, (1959): 177–183.

[20] Motzok, I., and Wynne, A. M.: Studies on the Plasma Phosphatase of Normal and Rachitic Chicks. 1. General Characteristics of the Enzyme. Biochem. J., 47, (1950): 187–193.

[21] Peterson, W. J., and Parrish, D. B.: Fluctuations of Phosphatase and Inorganic Phosphorus in the Blood of the Laying Hen During the Period of Egg Formation. Poult. Sci., 18, (1939): 54–58.

[22] Peterson, W. J., and Parrish, D. B.: Phosphatase and Inorganic Phosphorus in the Plasma and Whole Blood of the Fowl. Poult. Sci., 18, (1939): 59–62.

[23] Reiner, J. M.: Behavior of Enzyme Systems. Burgess Publishing Co., Minneapolis, Minn., 1959.

[24] Reitman, S., and Frankel, S.: A Colorimetric Method for the Determination of Serum Glutamic Oxalacetic and Glutamic Pyruvic Transaminase. Am. J. Clin. Path., 28, (1957): 56–63.

[25] Rubinstein, D., and Denstedt, O. F.: The Metabolism of the Erythrocyte. III. The Tricarboxylic Acid Cycle in the Avian Erythrocyte. J. Biol. Chem., 204, (1953): 623–637.

[26] Siegel, A., and Bing, R. J.: Plasma Enzyme Activity in Myocardial Infarction in Dog and Man. Proc. Soc. Exper. Biol. Med., 91, (1956): 604–607.

[27] Sigma Chemical Co.: Determination of Serum Acid and Alkaline Phosphatase and "Prostatic" Acid Phosphatase. Sigma Tech. Bull. 104, St. Louis, Mo., 1957.

[28] Sigma Chemical Co.: A Simplified Method for the Clinical Determination of Serum Glutamic-Oxalacetic Transaminase and Serum Glutamic-Pyruvic Transaminase at Approximately 500 mμ. Sigma Tech. Bull. 505, St. Louis, Mo., 1958.

[29] Snedecor, G. W.: Statistical Methods. 5th ed. Iowa State College Press, Ames, 1956.

[30] Stutts, E. C., Briles, W. E., and Kunkel, H. O.: Plasma Alkaline Phosphatase Activity in Mature Inbred Chickens. Poult. Sci., 36, (1957): 269–276.

[31] Tanabe, Y., and Wilcox, F. H.: Effects of Age, Sex and Line on Serum Alkaline Phosphatase of the Chicken. Proc. Soc. Exptl. Biol. & Med., 103, (1960): 68–70.

[32] Thiers, R. E., and Vallee, B. L.: Analytical Considerations in Serum Enzyme Determinations. Ann. New York Acad. Sci., 75, (1958): 214–226.

[33] Wacker, W. E. C., Ulmer, D. D., and Vallee, B. L.: Metalloenzymes and Myocardial Infarction. II. Malic and Lactic Dehydrogenase Activities and Zinc Concentrations in Serum. New England J. Med., 255, (1956): 449–456.

[34] Wiese, A. C., Johnson, B. C., Elvehjem, C. A., Hart, E. B., and Halpin, J. G.: A Study of Blood and Bone Phosphatase in Chick Perosis. J. Biol. Chem., 127, (1939): 411–420.

[35] Wroblewski, F., and LaDue, J. S.: Lactic Dehydrogenase Activity in Blood. Proc. Soc. Exptl. Biol. & Med., 90, (1955): 210–213.

[36] Wroblewski, F., and LaDue, J. S.: Serum Glutamic Pyruvic Transaminase in Cardiac and Hepatic Disease. Proc. Soc. Exptl. Biol. & Med., 91, (1956): 569–571.

SUMMARIO IN INTERLINGUA

Nivellos del Activitate Enzymatic in le Plasma de Gallinas

Esseva investigate le applicabilitate de varie technicas de essayage de enzymas a plasmas avian. Modificationes del sequente technicas es appropriate: Le methodo de Wróblewski e LaDue pro dishydrogenase lactic (DHL), le methodo de Siegel e Bing pro dishydrogenase malic (DHM), le methodo de Karmen pro transaminase glutamic-oxaloacetic (TGO), le methodo de Reitman e Frankel pro transaminase glutamic-pyruvic (TGP), e le methodo de Bessey *et al.* pro phosphatase acide e pro phosphatase alcalin (PH-acide, PH-alc).

Le nivellos del enzymas esseva mesurate in plasma ab normal unicrestate leghorns blanc e plymouth-rocks blanc de inter 5 septimanas e 6 menses de etate. Le nivellos medie de TGO, TGP, e PH-acide del gallinas in le varie gruppos de etate esseva simile. Le nivellos medie de DHL, DHM, e PH-alc esseva plus basse aves adulte que in aves de etates plus juvene. Nulle statisticamente significative differentias esseva observate inter le nivellos plasmatic medie de TGO, TGP, DHL, e DHM in gallos e gallinas o inter le nivellos plasmatic medie de TGO, TGP, DHL, DHM, e PH-acide in le un e le altere del duo racias.

Epizootic Hemorrhagic Disease in White-Tailed Deer—Characteristics of the South Dakota Strain of Virus

E. C. PIRTLE, Ph.D., and J. M. LAYTON, M.D.

Vermillion, South Dakota, and Iowa City, Iowa

A HIGHLY FATAL disease of deer that occurred in New Jersey in the late summer and early fall of 1955 was described by Shope *et al.*[3] The disease was characterized by the appearance of small hemorrhages in skin and subcutaneous areas and of varying degrees of hemorrhage involving the abdominal viscera, esophagus, trachea, and heart. Straw-colored fluid was observed in the peritoneal cavity and pericardial sac. The disease was produced in deer given inoculations of supernatant fluid or bacteria-free filtrates derived from infected tissue. Attempts to produce the disease in several other species of animals were not successful. All evidence obtained from the preliminary studies[3] pointed to a viral cause for this disease.

A similar epizootic was reported by Fay *et al.*[1] in white-tailed deer in Michigan in the fall of 1955. They found that supernatant fluid and bacteria-free filtrates of infected deer tissue were infective for healthy deer but not for mice, rabbits, hamsters, lambs, hares, or a red squirrel.

In a later article, Shope *et al.*[4] reported that rabbits, adult and newborn mice, guinea pigs, pups, hamsters, swine, calves, and sheep failed to develop signs of illness when inoculated by several routes with deer-lethal tissue preparations. The virus did not propagate in embryonating eggs or deer kidney tissue culture but did retain its infectivity for deer at least nine months when stored on dry-ice. The virus was not transmitted from diseased to healthy deer by direct contact. The designation "epizootic hemorrhagic disease of deer (EHD)" was proposed to identify this disease in white-tailed deer.

There is no record that this disease occurred in South Dakota's deer population during the same year it appeared in New Jersey and Michigan. One year later, however, in the late summer and early fall of 1956, an epizootic did occur in white-tailed deer in southeastern South Dakota. Both white-tailed deer *(Odocoileus virginianus)* and mule deer *(Odocoileus hemionus)* comprise South Dakota's deer population, with the former predominating. Mule deer did not appear to be involved in the epizootic. Shope *et al.*[3] found that, even though the New Jersey and South Dakota strains of hemorrhagic disease virus induced similar lesions, they were different immunologically.

During the past three years, continuing studies have been carried out to investigate various aspects of experimental epizootic hemorrhagic disease and the virus associated with it. This report deals with some important characteristics of the South Dakota strain of hemorrhagic disease virus.

MATERIALS AND METHODS

Experimental Infection of Deer.—The South Dakota Game Department provided white-tailed deer (WTD) and mule deer fawns that were trapped in the Black Hills in western South Dakota. Infection was accomplished by inoculating WTD intramuscularly with 3.0 ml. of supernatant fluid from a 10 per cent suspension of infected deer spleen. Suspensions were prepared in sterile brain-heart infusion broth (Difco).

Source of Virus.—The South Dakota strain of epizootic hemorrhagic disease (EHD) virus was used throughout this investigation and was obtained from spleens of experimentally infected WTD. Crude preparations of infected WTD spleen were employed as beginning material in experiments, to be described, that were designed to study the characteristics of EHD virus.

Vaccine for Immunization of WTD.—Supernatant fluid from a 40 per cent suspension of infected WTD spleen prepared in 0.3 per cent formalized phosphate-buffered saline solution was incubated

Received for publication April 22, 1960.

From the Department of Microbiology and Public Health, School of Medicine, State University of South Dakota, Vermillion; and the Department of Pathology, College of Medicine, State University of Iowa, Iowa City.

The South Dakota Game Department and the authors express their thanks to Dr. Richard E. Shope for originally passaging the South Dakota deer agent and for supplying infected deer tissues in order that the present studies might be initiated.

This investigation was supported in part by the Game Research Division, Department of Game, Fish and Parks, Pierre, S.D.

at 20 C. for three days and then was kept at 4 C. until used as vaccine. Two yearling bucks were given three injections of vaccine at weekly intervals, 1.0 ml. initially, followed by 2.0 ml. and 3.0 ml., respectively. Serums obtained two weeks after the third inoculation were tested for ability to neutralize EHD virus in WTD.

Stability of EHD Virus.—Splenic tissue and supernatant fluid prepared from spleen suspensions were stored at 4 C. to −60 C. Suspensions of spleen were prepared in either sterile infusion broth, broth containing a 50 per cent final concentration of glycerin,* or Hanks' balanced salt solution (BSS). Supernatant fluid, prepared from spleen tissue immediately after thawing, and stored supernatant fluid were tested for their ability to produce typical EHD in experimental WTD.

Host Range of EHD Virus.—Spleen supernatant fluid lethal to WTD was inoculated by various routes into adult and suckling Webster strain mice, adult and newborn Hartley strain guinea pigs, young ferrets (Manor Farms), weanling goats, and young albino rabbits. Three blind passages were carried out at six-day intervals in the species mentioned, and tissue preparations from the third passages in goats and ferrets were tested in WTD. Single injections with infected spleen inoculum were given to 4 mule deer fawns and 1 opossum *(Didelphis marsupialis* var. *virginiana)*. Finally, EHD virus-infected WTD tissues were fed to snapping turtles *(Chelydra serpentina)*.

Inoculation of Tissue Cultures with EHD Virus.—Cultures of cells derived from fetal WTD spleen were initiated in this laboratory, using medium 199 (Difco), 85 per cent, and normal deer serum, 15 per cent. Representative cultures were inoculated in duplicate with one of the following: (1) EHD virus-infected spleen supernatant fluid, (2) virus plus EHD-immune deer serum, (3) virus plus normal deer serum, (4) normal deer spleen supernatant fluid, (5) EHD-immune deer serum only, and (6) normal deer serum only. The six groups of initial cultures were incubated at 36 C. for 96 hours. Fluids harvested from the initial cultures, and from each of the three additional passages made at four-day intervals, were diluted 10^{-2} in Hanks' balanced salt solution for inoculation of succeeding tissue culture passages. Pooled harvest fluids from the fourth tissue culture passage of EHD virus-infected spleen supernatant fluid were inoculated into 1 WTD.

Purification of EHD Virus.—The fluorocarbon deproteinization method described by Gessler *et al.*[2] was employed to obtain EHD virus from infected spleen as a relatively hemoglobin-free aqueous preparation. Aliquots of the aqueous preparation, which produced typical EHD in WTD, were filtered through sintered glass filters of U.F. porosity, sprayed[5] onto grids coated with polyvinyl formal resin,** shadowed with chromium, and observed with an RCA EMU-3E electron microscope.

Graded Filtration of EHD Virus.—Aliquots of sintered glass filtrates were filtered through ultrafine membrane filters* under 12 atmospheres of N pressure, first through a coarse filter of 100 to 35 mμ average pore size, and then through a medium filter of 35 to 20 mμ average pore size. The sintered glass filtrates and coarse and medium membrane filtrates were inoculated into individual WTD to determine whether they would produce typical EHD.

RESULTS

Gross Pathology of Experimental EHD.—Following inoculation with the South Dakota strain of EHD virus, 38 WTD developed signs of illness between days 6 and 8. With onset of clinical signs, they became listless, progressively weaker and finally comatose. Only 1 deer was observed to recover. The interval from appearance of signs to death varied from 12 to 24 hours. Death appeared to be due to profound shock.

Variable numbers of petechiae and ecchymoses in the axillary and inguinal areas were seen upon external examination immediately after death. Numerous ecchymoses in the subcutaneous tissue were a constant finding.

Although the degree was variable, retroperitoneal edema was observed in every animal examined. Yellow gelatinous exudate adhered to the omentum, and 50 to 850 ml. of yellow fluid exudate was found free in the peritoneal cavity. Similar exudate was also found free in the pleural cavities and in the pericardial sac. Leukocytes and erythrocytes were numerous in this exudate which clotted within 30 minutes at room temperature. Gross hemorrhage and edema were observed regularly in the gastrointestinal tract, spleen, kidneys, nasal mucosa, trachea, and lungs and were observed usually in the heart, thymus, adrenals, and pituitary. Gross evidence of necrosis was rarely observed.

Viability of EHD Virus.—Supernatant fluid was prepared from freshly thawed splenic tissue that had been stored for two months to two years at either −20 C. or −60 C. and had been checked on several occasions for ability to produce typical EHD. A total of 9 WTD were successfully infected with supernatant fluid from spleen stored at −60 C. for two years, and 6 WTD were infected with spleen stored at −20 C. for 14 months.

* Merck, reagent grade, Merck & Co., Inc., Rahway, N.J.

**Formvar, produced by Shawinigan Resins Corp., Springfield, Mass.

* Schleicher and Schuell Co., Keene, N.H.

It was desired to learn whether the EHD virus in a given supernatant fluid prepared from infected spleen would retain its viability and, thus, serve as a reference deer-lethal inoculum for a period of one month. A 20 per cent suspension of infected spleen was prepared in sterile infusion broth and centrifuged lightly to remove particulate matter. Aliquots of the supernatant fluid were diluted 1:2 in sterile broth, Hanks' BSS, and glycerin. The broth and one BSS preparation were stored in rubber-capped vials at 0 C., and the glycerin and another BSS preparation were stored in similar containers at 4 C. After seven weeks' storage, the four preparations were tested for suitability as reference inoculums by inoculating 3.0 ml. intramuscularly into separate WTD. Each of the inoculums produced typical EHD with characteristic features of the disease observed at necropsy.

Neutralization of EHD Virus with Serum from Immunized and Recovered Deer.—It was mentioned under gross pathologic findings that only 1 WTD recovered from experimental EHD. Three weeks after recovery, serum was obtained from this deer; and this serum, as well as the two serum samples obtained from yearling bucks vaccinated with formalized vaccine, was tested for neutralizing antibodies against EHD virus. The two bucks were not challenged with virulent virus. Each serum was mixed with an equal volume of 20 per cent spleen supernatant fluid and held at room temperature for two hours before injection of 3.0 ml. of each serum-virus mixture into each of 2 WTD. Each animal in a specific group was given an additional 5.0 ml. of the same serum as that in the mixture administered. Two control animals were given equivalent injections of virus alone, and these died on day 8. The 2 WTD given EHD virus plus serum from the recovered WTD survived. The incubation period was prolonged to the twelfth day in deer of the two groups given serum from the bucks vaccinated with formalized vaccine, but all were dead by the fifteenth day after injection with the serum-virus mixtures.

Infectivity of South Dakota EHD Virus for Species Other Than WTD.—No signs of overt illness were observed in goats, ferrets, rabbits, mice, or guinea pigs following the initial inoculation or during three blind passages in these species.

Thirty per cent suspensions in sterile broth were prepared from the lungs of the ferrets and from the spleen of the goat used for the third passage. Four milliliters of supernatant fluid from these suspensions was inoculated into separate WTD. These 2 WTD, having no signs of EHD during three weeks' observation, were given challenge doses of deer-lethal inoculum. The possibility was considered that EHD virus had been carried through the passages in ferrets and goats, and that loss of lethality but retention of immunogenicity for WTD had resulted. This was obviously not the case, however, since both WTD succumbed to challenge on day 8.

Four mule deer and 2 WTD controls were inoculated intramuscularly with an infected spleen inoculum. The 2 controls died on day 9. Three of the mule deer remained free of signs during several weeks' observation, but the fourth died on day 12. The necropsy findings in this mule deer were not typical of those observed on numerous occasions in WTD, but minimal evidence of hemorrhage was observed on the surface of the abomasum, left adrenal, and right atrium and left ventricle of the heart. There was a small amount of yellow fluid exudate in the pericardial sac but none in pleural or peritoneal cavities.

The opossum and snapping turtle remained free of signs during five weeks' observation.

Propagation of EHD Virus in Cultured Cells from Fetal Deer Spleen.—No cytologic alterations were observed in cells of the initial tissue cultures of the six groups inoculated as described earlier. However, in the second, third, and fourth passages of the harvest fluids from initial cultures inoculated with infected spleen supernatant fluid alone, progressive degeneration of culture cells was observed. No alterations were observed in the cells of cultures in which infected spleen supernatant fluid-EHD immune deer serum was passaged. Normal deer serum did not prevent the cytopathic effect. The other three series of cultures comprising control groups remained normal in appearance throughout four passages. Mule deer serum was not tested.

Only 1 WTD was available for testing the harvest fluids from the fourth tissue culture passage of infected spleen inoculum. The test animal remained free of signs for 20 days but died in shock on the following day and was found to have the minimal

gross lesions of EHD. It had been necessary to store the fourth passage fluids for two months at −20 C. until the single WTD became available.

Size of South Dakota EHD Virus Determined from Electron Microscopic and Filtration Studies.—After determining that viable EHD virus could be obtained as a partially purified preparation from infected spleen by two treatments with fluorocarbon, a sintered glass filtrate of one such preparation was examined with the electron microscope as already described. Particles with a relatively spherical appearance were observed, and their size was calculated to range from 20 to 30 mμ in diameter.

In corroboration of the results obtained by electron microscopy, it was determined that the sintered glass and the graded membrane filtrates produced typical EHD upon inoculation into separate WTD. Thus, there was relatively good agreement between the size of the particles observed with the electron microscope and the average pore size of 35 to 20 mμ, the finest filter used that allowed passage of the EHD virus.

Discussion

Only 1 of the 38 experimentally infected WTD recovered from overt EHD. Shope *et al.*[4] found that the South Dakota strain of EHD virus produced fewer fatal infections in New Jersey WTD than did the New Jersey strain. This perhaps reflects susceptibility differences in geographically separated WTD.

The rapidity with which infected WTD succumb following onset of signs is most striking. The widespread occurrence of hemorrhage and edema and the accumulation of exudate are indicative of generalized damage to the blood vascular compartment, probably the capillaries.

It was found that the South Dakota strain of EHD virus, if stored as infected spleen, retains its capacity for producing typical EHD for two years at −60 C. and for at least 14 months at −20 C. The stability of the virus for at least seven weeks in supernatant fluid held at 4 C. is likewise important, since it makes possible the employment of reference inoculums for at least one month.

Serum obtained from a WTD that had recovered from experimental EHD contained antibodies which protected normal WTD from a lethal dose of virus. Serums from deer that were actively immunized with formalin-inactivated EHD virus, although apparently prolonging the incubation period, failed to protect test deer from typical EHD and death. The latter findings suggest that (a) the inactivated vaccine contained too little viral antigen, (b) the formalin treatment brought about undesirable alteration of the immunizing antigen[s], or (c) larger doses of vaccine should have been given for a longer period of time. Additional studies will be required to elucidate these possibilities.

Of the non-deer species inoculated with EHD virus-infected material, none developed signs of illness, either upon initial inoculation or following three blind passages. The third-passage material from ferrets and goats failed to infect or to elicit an immunogenic response when inoculated into test deer, suggesting that EHD virus did not survive during passaging in these non-deer species.

Mule deer do not share with WTD the high susceptibility to experimental infection with South Dakota EHD virus. This evidence corroborates the findings in nature that mule deer were not involved in the 1956 epizootic of EHD in South Dakota WTD.

Cultured cells of fetal deer spleen remained normal in appearance for 96 hours after inoculation with EHD virus. However, a cytopathic effect involving gradual degeneration of cells in all subsequent tissue culture passages was observed. The cytopathic effect did not occur in comparable cultures containing EHD-immune deer serum but did occur in normal deer serum-virus cultures. Control cultures remained normal. The single test deer inoculated only with the harvest fluids from the fourth tissue culture passage of virus died on day 21. Because this long an interval from inoculation to death in deer-to-deer passage of the virus had not been observed before, it is possible that the tissue culture material contained a minimal infective quantity of virus or that the virus lost some virulence during passaging.

The sizes of South Dakota EHD virus determined by filtration through membrane filters of known average pore size and by electron microscopy are in relatively close agreement and place it, with its range from 20 to 30 mμ in diameter, among the smaller viral agents.

Summary

Several characteristics of the South Dakota strain of virus causing epizootic

hemorrhagic disease of deer have been investigated. The virus has a signal pathogenicity for native white-tailed deer, but mule deer are relatively refractory to its effects. Adaptation of the virus to non-deer species was not successful, but a cytopathic effect in cultured fetal deer spleen cells was observed, beginning with the second tissue culture passage. Based on filtration experiments and electron microscopic studies, the virus was determined to range from 20 to 30 mμ in diameter and to be relatively spherical.

References

[1] Fay, L. D., Boyce, A. P., and Youatt, W. G.: An Epizootic in Deer in Michigan. Trans. 21st North Am. Wildlife Conf., (1956): 173–184.

[2] Gessler, A. E., Bender, C. E., and Parkinson, M. C.: 1. A New and Rapid Method for Isolating Viruses by Selective Fluorocarbon Deproteinization. New York Acad. Sci., 18, (1956): 701–702.

[3] Shope, R. E., MacNamara, L. G., and Mangold, R.: Deer Mortality—Epizootic Hemorrhagic Disease of Deer. New Jersey Outdoors, 6, (1955): 17–20.

[4] Shope, R. E., MacNamara, L. G., and Mangold, R.: A Virus-Induced Epizootic Hemorrhagic Disease of the Virginia White-Tailed Deer (*Odocoileus virginianus*). J. Exptl. Med., 111, (1960): 155–170.

[5] Williams, R. C.: A Method of Freeze-Drying for Electron Microscopy. Exptl. Cell Res., 4, (1953): 188–201.

SUMMARIO IN INTERLINGUA

Morbo Hemorrhagic Epizootic in Cervos Albi-Caudate (*Odocoileus virginianus*).

Characteristicas del Racia Sud-Dakota del Virus

Esseva investigate plures del characteristicas del racia Sud-Dakota del virus que causa morbo hemorrhagic epizootic de cervos. Le virus es signalmente pathogene pro autochthone cervos albi-caudate (*Odocoileus virginanus*), sed cervos mulo (*Odocoileus hemionus*) es relativemente resistente contra su effectos. Le adaptation del virus a species altere que cervos non succedeva. Nulle effecto cytopathic esseva observate in vitro in cellulas de fetal splen de cervo a partir del secunde passage histocultural. Basate super experimentos de filtration e studios de microscopia electronic, il es possibile asserer que le diametro del virus es in le ordine de inter 20 e 30 mμ e que su conformation es relativemente spheric.

Studies in Anaplasmosis. II. Electron Microscopy of *Anaplasma marginale* in Deer

MIODRAG RISTIC, D.V.M., Ph.D., and A. M. WATRACH, M.R.C.V.S., Ph.D

Urbana, Illinois

DEER are known to be susceptible to *Anaplasma marginale* infection,[1] and evidence has been presented recently to indicate the existence of well-advanced host-parasite adaptation and of a carrier stage in these animals.[2,3] Thus, deer are recognized as silent reservoirs of *A. marginale* infection and they may be responsible for the sudden appearance of epizootics in areas generally considered free of anaplasmosis.[4,13]

Investigations of morphologic features of *A. marginale* in infected bovine erythrocytes by means of electron microscopy have been conducted by several workers.[5,7,8,15] The methods employed, however, did not provide information concerning the fine internal structure of the organism. The present report concerns the morphologic characteristics and structural configuration of *A. marginale* in deer erythrocytes viewed in the electron microscope in shadow-cast preparations and ultrathin sections.

MATERIAL AND METHODS

Each of 3 splenectomized white-tail deer *(Odocoileus virginianus)*, approximately 1 year of age, were inoculated intravenously with 30.0 ml. of heparinized (0.5 mg. heparin sodium/1.0 ml. of blood) whole blood from an 8-month old, anaplasmosis carrier calf. The blood was considered satisfactory for electron microscopic examination when at least 20 per cent of the erythrocytes contained marginal Anaplasma bodies. The technique used to dehemoglobinize infected erythrocytes and study the organism as a three-dimensional structure has been described.[15]

For investigations of internal structure, the infected blood was fixed at 0 C. for 30 minutes in a 2 per cent aqueous solution of potassium permanganate buffered with acetate veronal at pH 7.2.[1] This was accomplished by placing approximately 4 to 5 drops of blood, drawn from the jugular vein by means of an 18-gauge needle, directly into 2 ml. of the fixative. To permit rapid penetration of the fixative, the tubes containing blood were gently agitated for five to ten seconds. Subsequently, the specimens were dehydrated in ethyl alcohol of ascending grades (20, 35, 70, and 95%) at 0 C. for 30 minutes in each and in absolute ethyl alcohol with three changes for one hour. The preparations were then infiltrated with monomeric methacrylate (5:2 n-butyl and methyl) for two hours and embedded in prepolymerized methacrylate. Following polymerization for 48 hours under ultraviolet light at 30 C., the specimens were sectioned with a Porter-Blum ultramicrotome equipped with a diamond knife. All sections were mounted on "Athene" grids * coated with a collodion film stabilized by carbon[21] and examined with an RCA, EMU 3 c electric microscope.

Blood films were stained with fluorescein-labeled anti-Anaplasma globulin[10] for serologic identification of the marginal bodies. Anti-Anaplasma globulin was secured from the pooled antiserums of 5 experimentally infected calves in which the complement-fixing antibody titers ranged from 1:160 to 1:640.

RESULTS

In preparations stained with fluorescein-labeled anti-Anaplasma globulin and examined with the ultraviolet light microscope, the marginal Anaplasma bodies appeared as brilliant yellow-green, sharply defined, round structures, measuring 0.5 to 1.4 μ in diameter (fig. 1). Approximately 70 per cent of these bodies occupied a marginal position in the erythrocyte, while the remaining ones were found dispersed throughout the cytoplasm, evidently without constant localization. Occasionally, an Anaplasma body was found situated at the tip of sickle-shaped erythrocytes. A maximum of 5 marginal bodies was observed within a single red blood cell.

Shadow-cast preparation of infected erythrocytes revealed two distinct morphologic forms of Anaplasma organisms. These were (1) classical marginal bodies and (2) initial bodies. The marginal body consisted

Received for publication July 7, 1960.

From the Department of Pathology and Hygiene, College of Veterinary Medicine, University of Illinois, Urbana.

This investigation was supported in part by research grant E-3315 from the National Institute of Allergy and Infectious Diseases, United States Public Health Service.

The authors thank Mrs. Sharon Dinger for technical assistance in preparing the blood specimens.

* Produced by Smethurst High-Light Ltd., Bolton, England.

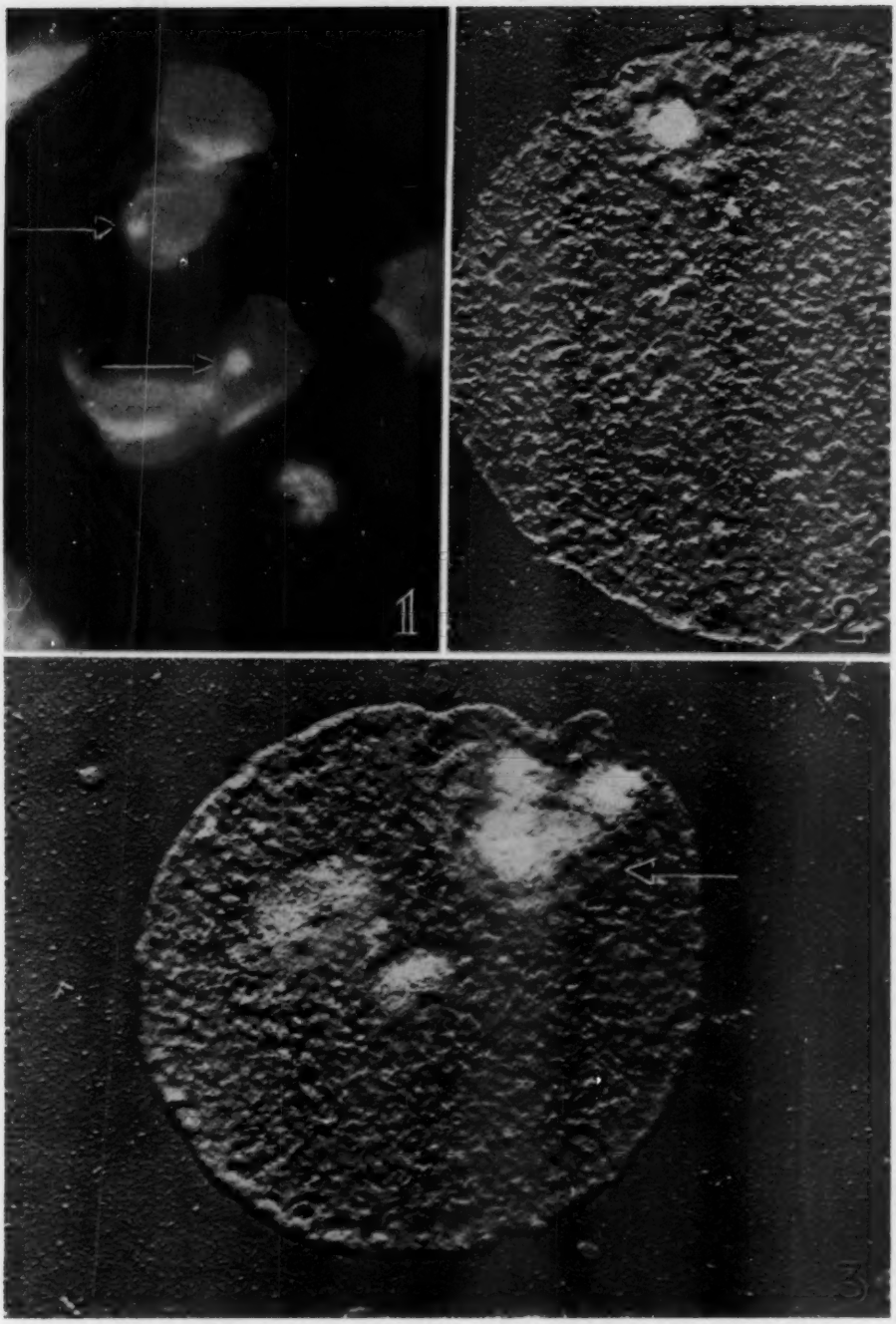


Fig. 1—Two marginal Anaplasma bodies stained with fluorescein-labeled anti-Anaplasma globulin and examined with ultraviolet microscope. x 1,800.

Fig. 2—Shadow-cast preparations of infected erythrocytes with marginal Anaplasma body consisting of single initial body. x 21,000.

Fig. 3—Marginal Anaplasma body consisting of 5 initial bodies embedded in outer, less dense matrix. The latter structure is indicated by arrow. x 21,000.

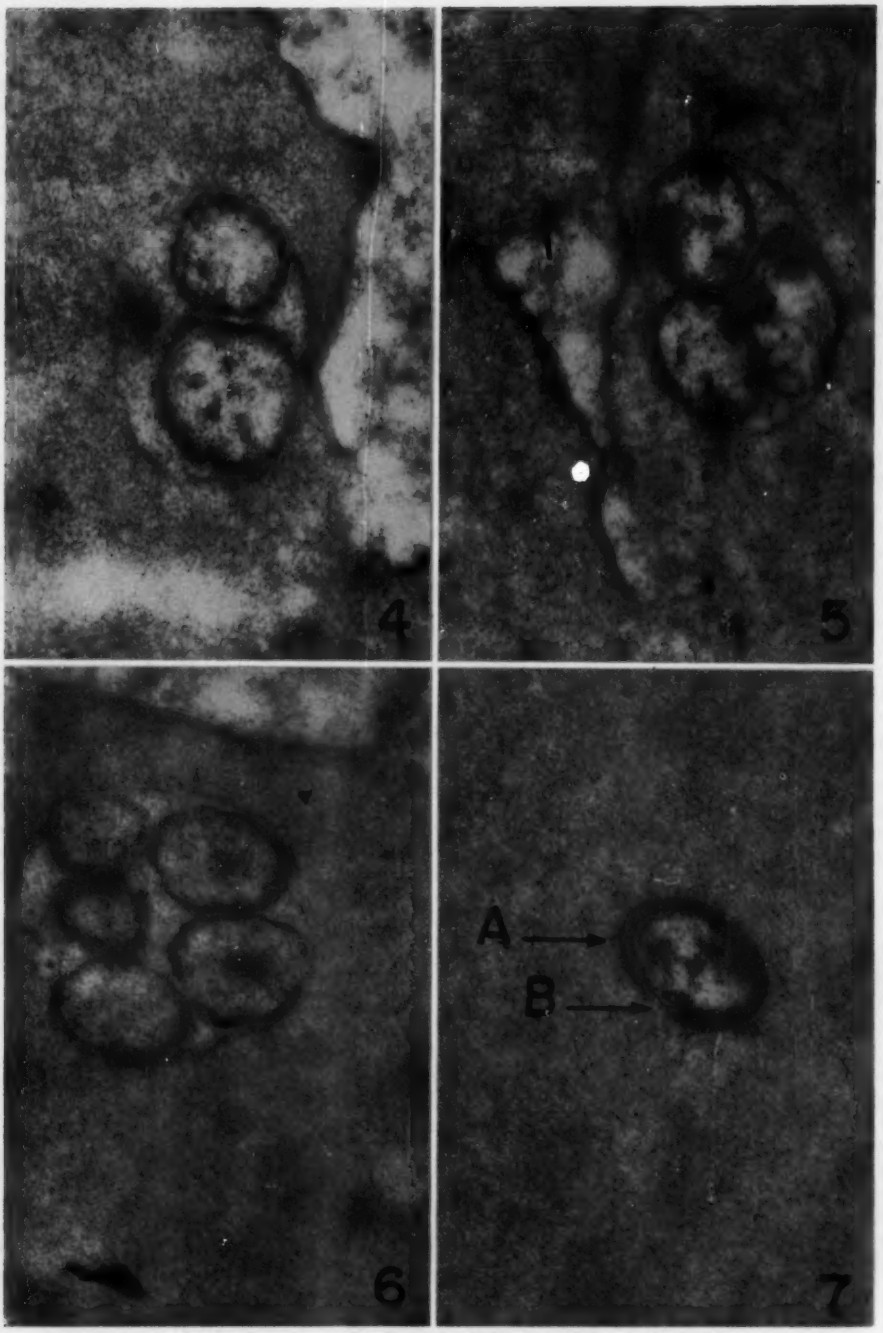


Fig. 4-6—Sectioned erythrocytes containing marginal Anaplasma bodies with 2, 3, and 5 initial bodies, respectively. The outer matrix is demarcated from the cytoplasm of erythrocytes by a fine membrane. x 55,000.

Fig. 7—Single initial Anaplasma body. Notice the outer matrix (arrow A) and two well defined membranes of this body, (arrow B). The inner structure of initial body consists of several dense aggregates of finely granular material embedded in electron-lucid plasma. x 55,000.

of an outer matrix in which a variable number (usually 1 to 8) of initial bodies, each approximately 0.4 μ in diameter, were situated (fig. 2, 3). With the exception of a slightly flattened appearance, the morphology of initial bodies resembled Anaplasma organisms which occurred in bovine erythrocytes.[15]

Viewed in ultrathin sections, the marginal body (fig. 4-6, 8) appeared separated from the cytoplasm of the erythrocyte by a limiting membrane approximately 10 mμ in thickness. The electron density and thickness of this membrane were comparable to those of the plasma membrane of the red cell. The outer matrix of the marginal body appeared less dense than the cytoplasm of the erythrocyte.

The initial bodies, representing the subunits of the marginal body, were round or oval and measured approximately 0.3 to 0.4 μ in diameter (fig. 5, 6). They were enclosed in two well-defined, dense membranes, each approximately 4 to 6 mμ in thickness. The space between these membranes was 10 to 30 mμ wide. The inner structure of the initial body consisted of one central and several peripheral dense aggregates of finely granular material embedded in an electron-lucid substance, for which the term "plasma" is proposed. The aggregates appeared to be connected with each other by narrow bands of a similar dense material (fig. 7).

Singly occurring initial bodies were also observed (fig. 7). They were surrounded by a narrow zone of relatively dense matrix, approximately 40 to 50 mμ in width. This zone was demarcated from the cytoplasm of the erythrocyte by a membrane similar to that of the marginal body.

An apparent multiplication of the initial body by binary fission can be seen (fig. 8, 9). The primary stage of this process is manifested as a constriction of the membranes. It is then followed by separation of the plasma and formation of two daughter forms. An apparently predeterminal fission stage, wherein two daughter forms are still connected by a narrow band of the plasma, is depicted (fig. 10).

DISCUSSION

As a result of fixation with potassium permanganate, it has become possible to secure more detailed information concerning structural organization of the *A. marginale*. In addition, preliminary elucidation of the mode of multiplication of the initial body has been possible. In an earlier report,[15] justification was offered for the term "initial body"; this body probably represents the infective form of the causative agent of anaplasmosis. Morphologic and structural characteristics of the initial body resemble those of the smallest bacteria and rickettsiae. The latter organisms possess a similar delimiting membrane and an undifferentiated cytoplasmic core.[14] However, inability of the initial Anaplasma body to grow in ordinary culture mediums and its requirement for intracellular multiplication relate some of its metabolic requirements closely to those of rickettsiae. While more data regarding the mechanism of multiplication of *A. marginale* are needed, it seems reasonable to assume, on the basis of preliminary observations, that the initial body multiplies by binary fission. This is in accordance with observations regarding the mode of multiplication of the members of the order Rickettsiales,[18] including the psittacosis-lymphogranuloma-venereum group of agents.[19] Further similarity between *A. marginale* and rickettsiae is indicated by common staining characteristics suggesting chemical resemblance, lack of disparity of content of desoxyribonucleic and ribonucleic acids,[12] pleomorphism, transmission by arthropods,[6] interference of Anaplasma with rickettsial Eperythrozoon,[9] sensitivity to a broad spectrum of antibiotics,[11] and propagation in cells such as erythrocytes, which have a low rate of metabolism.[15]

A number of modern biophysical, histochemical, and immunologic techniques have been used by various investigators in an attempt to define the morphology of *A. marginale* in a static state. Some of these techniques were staining of infected blood with fluorochrome dyes,[10] methylgreen pyronine and periodic acid-Schiff methods,[12] fluorescent antibody (including direct and indirect methods),[17] electron microscopy of ultrathin sections of the infected erythrocytes,[8,15] shadow-cast and replica preparations of infected erythrocytes,[5,15] and of the extracellular forms of Anaplasma.[15] None of these techniques revealed the presence of an accessory portion of the marginal body in the form of a flagellum or tail-like structure. The existence of such a flagellated *A. marginale* body, however, has been claimed recently.[7,20]

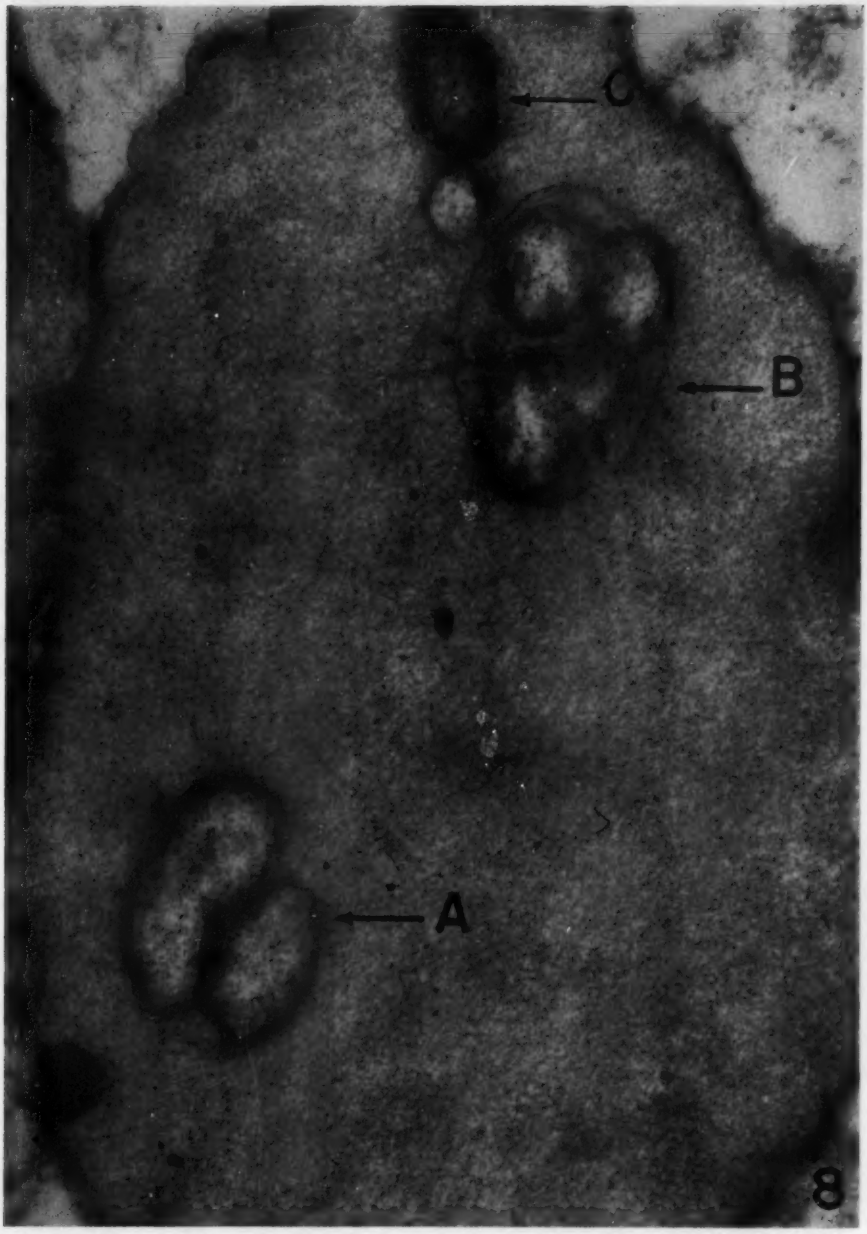


Fig. 8—Electron micrograph of infected erythrocyte with 2 marginal bodies (A and B), and a single body (C) apparently at the point of entering into erythrocyte. Marginal body at B contains an elongated and slightly constricted initial body evidently in process of division by binary fission.

Fig. 9—Early stage of multiplication of initial body by binary fission. The process is represented by median constriction of the body. x 55,000.

Fig. 10—Advanced stage of division of initial body. Notice narrow band of plasma by which two daughter forms are still connected. x 55,000.

The controversy regarding the true morphology of *A. marginale* is apparently due to the existence of an outer matrix surrounding both the marginal and the single Anaplasma bodies described. It is reasonable to believe that under the pressure of diffusing fluids at the time of hemolysis the less dense outer matrix might appear, when viewed by phase microscopy, as a flagellum-like structure projecting from the marginal body. The integrity of the outer matrix as a component of the *A. marginale* is indicated not only by its structural characteristics but also by tinctorial and antigenic properties, as demonstrated by staining with the Giemsa method and reaction to the specific, fluorescent antibody, respectively. The latter conclusion is based on the observation that *A. marginale* in deer are generally larger than those found in the bovine erythrocytes.

SUMMARY

Erythrocytes of deer with acute anaplasmosis have been studied in sections and shadow-cast preparations. The organism of *A. marginale* consisted of 1 to 8 initial bodies embedded in an apparently homogenous matrix. The latter was demarcated from the erythrocyte by a well-defined membrane approximately 10 mμ in thickness. The initial body was round or oval, 300 to 400 mμ in diameter, and enclosed in a double membrane. The internal structure of this body consisted of one central and a few peripheral, dense aggregates of finely granular material embedded in an electron-lucid substance. Preliminary observations suggest that the initial body may reproduce by binary fission.

References

[1] Boynton, W. H., and Woods, G. M.: Anaplasmosis Among Deer in the Natural State. Science, 91, (1940): 168.

[2] Christensen, J. F., Osebold, J. W., and Rosen, M. N.: Infection and Antibody Response in Deer Experimentally Infected with *Anaplasma marginale* from Bovine Carriers. J.A.V.M.A., 132, (April, 1958): 289–292.

[3] Christensen, J. F., Osebold, J. W., Harrold, J. B., and Rosen, M. N.: Persistence of Latent *Anaplasma marginale* Infection in Deer. J.A.V.M.A., 136, (May, 1960): 426–427.

[4] Christensen, J. F., Osebold, J. W., and Rosen, M. N.: The Incidence of Latent *Anaplasma marginale* Infection in Wild Deer in an Area Where Anaplasmosis is Enzootic in Cattle. Proc, 62nd Ann. Meet. U.S. Livestock San. A., (1959): 59–65.

[5] DeRobertis, E., and Epstein, B.: Electron Microscope Study of Anaplasmosis in Bovine and Red Blood Cells. Proc. Soc. Exptl. Biol. & Med., 77, (1951): 254–258.

[6] Dikmans, G.: The Transmission of Anaplasmosis. Am. J. Vet. Res., 11, (1950): 5–16.

[7] Espana, C., Espana, E. M., and Gonzales, D.: *Anaplasma marginale*. I. Studies with Phase Contrast and Electron Microscopy. Am. J. Vet Res., 20, (Sept., 1959): 795–805.

[8] Foote, L. E., Geer, J. C., and Stitch, Y. E.: Electron Microscopy of the Anaplasma body: Ultrathin Sections of Bovine Erythrocytes. Science, 128, (1958): 147.

[9] Foote, L. E., Levy, H., Torbert, B., and Oglesby, W. T.: Interference Between Anaplasmosis and Eperythrozoonosis in Splenectomized Cattle. Am. J. Vet. Res., 18, (July, 1957): 556–559.

[10] Gainer, J. H.: Technique for the Demonstration of *Anaplasma marginale* with the Fluorescent Dye, Acredine Orange. In press.

[11] Miller, J. G., Levy, H. E., Torbert, B. J., and Oglesby, W. T.: A Method of Screening Drugs to be Used in the Treatment of Anaplasmosis. Results of Testing with Aureomycin and Terramycin. Proc. Book, AVMA, (1952): 106–111.

[12] Moulton, J. E., and Christensen, J. F.: The Histochemical Nature of *Anaplasma marginale*. Am. J. Vet. Res., 16, (April, 1955): 377–380.

[13] Osebold, J. W., Christensen, J. F., Longhurst, W. M., and Rosen, M. N.: Latent *Anaplasma marginale* Infection in Wild Deer Demonstrated by Calf Inoculation. Cornell Vet., 49, (1959): 97–115.

[14] Plotz, H., Smadel, J. E., Anderson, T. F., and Chambers, L. A.: Morphological Structure of Rickettsiae. Exptl. Med., 77, (1943): 355–358.

[15] Ristic, M.: Structural Characterization of *Anaplasma marginale* in Acute and Carrier Infections. J.A.V.M.A., 136, (May, 1960): 417–425.

[16] Ristic, M., and White, F. H.: Detection of an *Anaplasma marginale* Antibody Complex Formed *in Vivo*. Science, 131, (1960): 987–988.

[17] Ristic, M., White, F. H., and Sanders, D. A.: Detection of *Anaplasma marginale* by Means of Fluorescein-Labeled Antibody. Am. J. Vet. Res., 18, (Oct., 1957): 924–928.

[18] Schaechter, M., Bozeman, F. M., and Smadel, J. E.: Study on the Growth of Rickettsiae. II. Morphologic Observations of Living Rickettsiae in Tissue Culture Cells. Virology, 3, (1957): 160–172.

[19] Sigel, M. M., Girardi, A. J., and Allen E. G.: Studies on the Psittacosis-Lymphogranuloma Group. I. The Pattern of Multiplication of Meningopneumonitis Virus in the Allantois of the Chick Embryo. J. Exptl. Med., 94, (1951): 401–413.

[20] Summers, W. A.: Reported at the Annual Meeting of the Anaplasmosis Research Workers, Gainesville, Fla., April, 1960.

[21] Watson, M. L.: The Use of Carbon Films to Support Tissue Sections for Electron Microscopy. J. Biophys. & Biochem. Cytol., 1, (1955): 183–184.

SUMMARIO IN INTERLINGUA

Studios in Anaplasmosis. II. Microscopia Electronic de *Anaplasma marginale* in Cervidos

Erythrocytos de cervido *(Odocoileus virginianus)* con anaplosmosis acute esseva studiate in sectiones e in preparatos a accentuation de umbra. Le organismo de *Anaplasma marginale* consisteva de 1 a 8 corpores initial includite in un matrice de character apparentemente

homogenee. Iste matrice esseva delimitate contra le erythrocyto per un nettemente definite membrana de un spissitate de approximativemente 10 mμ. Le corpore initial esseva ronde o oval. Su diametro mesurava 300 a 400 mμ. E illo esseva includite in un duple membrana. Le structura interne de iste corpore consisteva de dense aggregatos—un central e plures peripheric—de un finmente granular material que esseva includite in un substantia electrono-lucide. Observationes preliminari suggere que le corpore initial se reproduce possibilemente per un processo de fission binari.

The Etiology of Legume Bloat—Nonvolatile Acids

W. G. ROSEN, B.A., M.S., Ph.D.; HESTER FASSEL, B.S.;
R. E. NICHOLS, D.V.M., D.V.Sc., Ph.D.

Madison, Wisconsin

LEGUMES associated with bloat, particularly alfalfa, may contain compounds that, if present, occur in lesser amounts in forages that do not provoke bloat. Since preliminary experiments disclosed that aqueous extracts of alfalfa promoted rapid release of gas by fresh samples of ruminal contents, attempts were initiated to isolate, characterize, and evaluate the relative importance of the active constituents of alfalfa which affect the release of the gas.

MATERIALS AND METHODS

Samples of ruminal content were obtained by means of a sampling tube[16] from the bottom of the rumen of a healthy 7-year-old Jersey cow with a permanent ruminal fistula. The samples were strained through cheesecloth to remove large pieces of plant material. Conventional Warburg manometric techniques were employed to determine the effects of various addenda on the production of gas by these samples. For reasons stated by McBee,[14] activities of the addenda are expressed as changes in levels of the manometer fluid, ΔH (mm.).

Preparation of Extracts.—Extracts from hays were prepared by placing 200 Gm. of hay in 1 liter of distilled water; the mixture was then autoclaved at 15 lb. pressure for 15 to 30 minutes and the liquid was squeezed manually through cheesecloth and filtered. Extracts of alfalfa meal were prepared by placing 200 Gm. of alfalfa meal in 1 liter of distilled water. The meal-water mixture was permitted to stand at room temperature in the laboratory for 15 to 30 minutes with occasional stirring. It was squeezed through cheesecloth and the liquid was filtered.

Fresh alfalfa, bird's-foot trefoil, bluegrass, and brome grass were collected from the purest pasture stands locally available. They were ground in a meat grinder and their vacuolar juice was expressed by application of a pressure of 5,000 lb. per square inch in a Carver hydraulic press. Bluegrass and alfalfa yielded approximately 0.40 and 0.55 ml., respectively, of juice per gram of fresh weight. The juices were centrifuged to remove chloroplasts and other suspended matter. For studies during the winter months, freshly cut alfalfa was placed in plastic bags and stored at −10 C. As needed, the frozen material was thawed, ground, and its sap expressed as described.

Analyses.—For analysis, an aliquot of press juice or of water extract was adjusted to pH 2.0 with concentrated sulfuric acid. The resulting precipitate was removed by centrifugation. To remove lipoidal material, the supernatant fluid was extracted several times with petroleum ether. The acidified extract (usually 20 ml.) was placed in a Kutscher-Steudel extractor for extraction with diethyl ether.[20] After 17 hours or more of extraction, the ether layer was removed and a few milliliters of H_2O was added to it. The ether was then removed by distillation.

For assaying the gas-producing activity of this fraction, its volume was restored to 20 ml. with water. Although addenda were also tested at other pH values, a pH of 5.5 to 5.7 was selected for the assay standard because it is a representative value for expressed sap of plants.[26] At the conclusion of each experiment, the pH of the flask contents was determined. Generally, it was found that the pH had risen from an initial value of about 6.8 to 7.0, before tipping in the side-arm contents, to a final value of about 7.6 to 8.0. Control flasks, in which the addendum was distilled H_2O, were included in each experiment. The activities of fractions resulting from various chemical or physical treatments of extracts were always measured relative to the activity of the extract from which they were derived.

Paper Chromatography.—Chromatograms were prepared by spotting the material under study on Whatman No. 1 chromatographic filter paper. The papers were developed by ascending chromatography. The developer systems most frequently employed were (1) n-butanol:chloroform:formic acid, 88:10:12; (2) mesityl oxide:formic acid:water, 75:36:75 (shaken in a separatory funnel, and the aqueous phase discarded)[2]; and (3) phenol:water, 75:25 (w/v).

Papers were usually sprayed with a 0.05 per cent aqueous or ethanolic solution of bromeresol green adjusted with NaOH to a blue tint.[19] Acids appeared as yellow zones against a pale blue background.

Received for publication Aug. 31, 1959.

From the Department of Veterinary Science, College of Agriculture, University of Wisconsin, Madison, in conjunction with a state contribution project to the North Central Region cooperative research project NC-27, Causes and Control of Bloat in Ruminants. Published with the approval of the director of the Wisconsin Agricultural Experiment Station as paper No. NS 306. Dr. Rosen is now with the Department of Biology, Marquette University, Milwaukee, Wis., and Mrs. Fassel is with the Department of Zoology, Iowa State Univ., Ames.

Supported in part by funds from the Animal Disease and Parasite Research Division, ARS, USDA.

RESULTS

On comparable fresh and dry weight bases, the juice of fresh and frozen alfalfa caused the most marked stimulation of the production of gas in the Warburg vessels. Juice of fresh grass produced less, and aqueous extracts of alfalfa meal and alfalfa hay caused even less stimulation (fig. 1).

Activity of extracts was not markedly diminished by prolonged boiling, indicating that the active material was heat stable and nonvolatile. That the active materials were relatively small polar molecules was indicated by the fact that the activity was readily dialyzable.

Adjustment of juices or extracts to pH 2.0 with concentrated sulfuric acid resulted in the formation of a heavy, tan precipitate, with most of the gas-producing activity remaining in the soluble fraction. Fresh or acid-treated extracts retained their activity when stored at 4 C. The gas-producing activity was almost quantitatively retained (table 1) by strong anion exchange resins (IRA 400, IRA 410, Dowex 1) and by a mixed bed resin (Monobed MB-1) and was not retained by strong cation exchange resins (IR-120, Dowex 50).

TABLE 1—Distribution of Gas-Producing Activity of Alfalfa Meal Extract Following Various Fractionation Procedures

Addendum	△H (mm.)
Whole extract	115
Whole extract passed through cation exchange resin (IR 120)	77
Whole extract passed through anion exchange resin (IRA 410)	21
Supernatant fluid of whole extract after acid precipitation	90

The extracts, adjusted to pH 5.5 to 5.7, were added from side-arms to rumen fluid in Warburg vessels. Readings were made 50 minutes after the addition of side-arm contents.

Chromatograms of the supernatant fluid following acid precipitation contained several large, and in some cases incompletely resolved, acidic zones. When the ether and water fractions were chromatographed following ether extraction of this supernatant fluid, it was found that a number of well-defined spots of high Rf* appeared in the reconstituted ether fraction, while several poorly resolved spots of lower Rf remained in the aqueous fraction.

* Rf = ratio of the movement of the compound on the paper to the movement of the developing liquid on the paper.

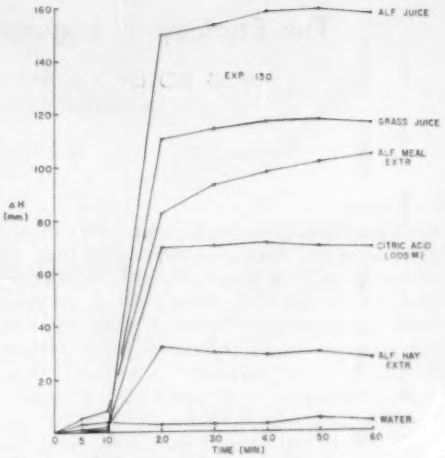


Fig. 1—Effects of forage extracts on gas production by bovine rumen fluid. The extracts, adjusted to pH 5.5 to 5.7, were added to the Warburg vessels from side-arms after the ten-minute reading.

Comparison of Rf values of the spots which appeared on chromatograms of the ether fraction with Rf values of known organic acids showed that they agreed closely with those of succinic, malic, malonic, and citric acids (fig. 2).

A striking difference between chromatograms of extracts of alfalfa as compared to chromatograms of extracts of bluegrass was that the former had a prominent spot, the Rf of which closely compared with that of malonic acid. This spot was small or entirely lacking in chromatograms of bluegrass extracts. Conversely, a spot with an Rf approximating that of succinic acid was relatively more abundant in chromatograms of grass extracts than in chromatograms of alfalfa extracts (fig. 2).

It was found that alfalfa extracts which had stood for some time in the refrigerator showed, when chromatographed, a large spot with an Rf closely approximating that of lactic acid. Since chromatograms of fresh extracts lacked this spot, it was presumed that lactic acid had accumulated as a result of microbial action during storage.

The addition of citric, malonic, or succinic acids (concentration, 0.005 M.) to diluted ruminal samples caused a marked increase in gas evolution by the samples (fig. 3). Gas evolution was largely obscured when KOH was placed in the center

wells of the Warburg vessels, indicating that the evolved gas was largely, if not entirely, CO_2.

Most of the evolution of gas was completed in ten to 15 minutes. With the addition of forage extracts, however, gradual evolution of gas was often observable for up to 60 minutes. The data reported here are limited to production of gas during the first 20 minutes.

Gas evolution was reduced when $AgNO_3$ or formalin were added to the vessels, suggesting that the evolved CO_2 was at least partly microbial in origin. Some of the evolved gas might have arisen from liberation of dissolved bicarbonate by the slightly acidic addenda. Various compounds added to a dilute bicarbonate solution from the

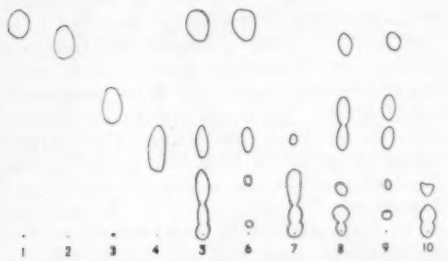


Fig. 2—Tracing of a chromatogram of reference acids and forage acids. (1) succinic acid; (2) malonic acid; (3) malic acid; (4) citric acid; (5) supernatant fluid following acid precipitation of expressed juice from bluegrass; (6) aqueous phase of the ether phase following ether extraction of fraction 5; (7) aqueous phase of fraction 5 remaining after ether extraction; (8-10) alfalfa, same fractions as 5-7.

sidearms of Warburg vessels caused gas evolution (fig. 4). This mechanism may be a source of a significant portion of the gas evolved by the ruminal samples when phosphate or organic acids are added to it.

Following ether extraction, 40 to 60 per cent of the activity appeared in the reconstituted ether fractions, while most of the remainder of the original activity remained in the aqueous fraction.

The compounds which were responsible for the portion of the gas-producing activity which is not extracted by ether have not been identified. A portion of this activity may be due to incomplete extraction of the carboxylic acids into the ether fraction. Chromatograms of the water residue

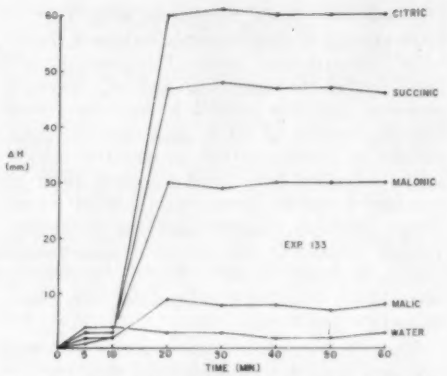


Fig. 3—Effects of organic acids on gas production by bovine rumen fluid. The acids, adjusted to pH 5.5 to 5.7, were added to the Warburg vessels from side-arms after the 10-minute reading. Water was added from the side-arm of the control vessel. Final acid concentration was 0.005 M.

following ether extraction, when developed with phenol and sprayed with ninhydrin, revealed ninhydrin-positive materials, suggesting amino acids.

DISCUSSION

The compounds which stimulated gas production are compounds which occur as constituents of ruminant feeds, and it is therefore reasonable to assume that the results *in vitro* may reflect an *in vivo* situation which could contribute to bloat.

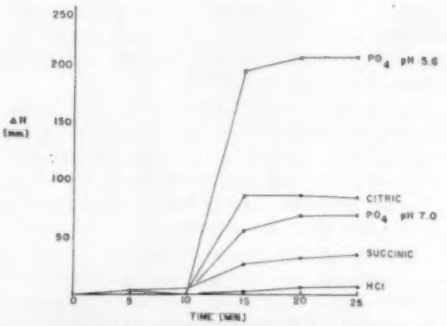


Fig. 4—Effects of various compounds on the release of carbon dioxide from bicarbonate. Main compartments contained 0.035 M. $NaHCO_3$ before tipping. Succinic and citric acids, and phosphate, were added at a final concentration of 0.005 M.; HCl was added at a final concentration of 0.05 M. Addenda were adjusted to pH 5.6, except where indicated.

Newbold [15] and Fergusen and Terry [5] have reported that legumes enhance the *in vivo* fermentation rate. Hungate *et al.*[8] have found that the addition of clover to ruminal contents results in an increase of gas production *in vitro*, and that the magnitude of fermentation is greater in ruminal samples from bloated cows than in ruminal samples from nonbloated cows. These findings suggest that the bloat-provoking capability of certain legumes may result, at least in part, from the relative abundance in these plants of factors which stimulate gas formation.

There is agreement between the observed differences in activity of grass and legume extracts and the relative abundance therein of the active organic acids. Turner and Hartman [25] reported that citric, malic, and malonic acids are present "in notable amount" in alfalfa. Bentley [1] found that citric and malic acids were present in concentrations of 1.0 to 4.0 mg. per gram of fresh weight in the leaves of 27 species of legumes, including alfalfa, and that malonic acid was present in concentrations of 0.5 to 2.0 mg. per gram of fresh weight in 18 species of legumes, including alfalfa. Except for two representatives of the Umbelliferae, other plant families either lacked malonic acid or contained only small amounts. Holton and Noll [7] reported that lupine was relatively rich in malonic acid; whereas, oats and barley contained small amounts and none was detected in peas. Pierce and Appleman [18] reported that, on a dry weight basis, alfalfa seedling leaves contained much greater amounts of oxalic, malic, and unknown acids, and somewhat smaller amounts of citric acid, than did bluegrass seedling leaves.

On both a fresh and dry weight basis, alfalfa appears to be richer in organic acids than grass is. Both the presence of malonic acid and the relative abundance of other organic acids may be of significance in the development of alfalfa bloat.

Results of this research do not permit a conclusion as to the manner in which the organic acids may cause evolution of gas in the rumen. Two mechanisms are suggested. The acids may act by causing release of CO_2 from the bicarbonate initially present in the rumen. That this is very likely the source of at least a significant portion of the evolved CO_2 is indicated by the experiment illustrated (fig. 4). A second mechanism by which CO_2 might be evolved is through the action of enzymes.[17]

The three organic compounds whose addition to rumen fluid caused the largest releases of gas are all carboxylic acids which either may serve directly as substrates for bacterial carboxylases or may be metabolized to a form which in turn may be decarboxylated.

Johns [10, 11] and Sijpesteijn and Elsden [21] have found that the ruminal bacterium, *Veillonella gazogenes*, is able to perform a rapid decarboxylation of succinic acid, resulting in the production of propionic acid and CO_2. Doetsch *et al.*[4] found that all the substrates which were found to be active in our tests are capable of rapid dissimilation by washed suspensions of ruminal bacteria. However, these workers acidified the reaction mixtures at the end of the experiments in order to release all dissolved CO_2 and, consequently, obtained different activities for the various acids. Citric acid has not, to our knowledge, been previously tested for its effects on gas production by ruminal microbes. Its activity may be the result of its direct decarboxylation [6] or of its conversion to an active substrate, *i.e.*, dissimilation to succinate.[12, 24] The activity of malonic acid is of particular interest. In addition to reports that it is directly decarboxylated by bacterial enzymes,[27] it may also exert an effect by inhibiting succinic dehydrogenase, thus "sparing" succinic acid for direct decarboxylation.[22]

The failure of some workers [13] to induce bloat consistently by administration of "physiologic" concentrations of saponins, and the success of others in inducing froth *in vitro* [26] with legume extracts, suggests that a combination of factors is required before bloat will occur. Johns [9] cites reports which indicate that the ruminal contents must be in a "requisite condition" before frothy bloat can be induced. A plausible interpretation of the situation is that froth will result from the feeding of compounds which enhance gas production only if the physiochemical factors needed for bubble retention are simultaneously present. Conversely, introduction of foam stabilizers into the rumen will result in bloat only if a source of gas is also present.

The present findings indicate that certain nonvolatile organic acids may be of significance in the gas-forming stage leading to frothy bloat.

Summary

Aqueous extracts and expressed juices of forage plants caused marked evolution of gas when added to samples of bovine ruminal contents in Warburg vessels. Gas production was greater with addenda prepared from alfalfa than with corresponding addenda prepared from bluegrass.

Chemical studies of these forage preparations indicated that 40 to 60 per cent of their gas-producing activity was attributable to three carboxylic acids, *i.e.*, citric, malonic, and succinic acids.

One-dimensional paper chromatography of fractions of the forage preparations indicated that the more active extracts of alfalfa contained malonic acid, which was absent from the less active extracts of bluegrass. The possible significance of these carboxylic acids, and particularly of malonic acid, in the etiology of legume bloat is discussed.

References

[1] Bentley, L. E.: Occurrence of Malonic Acid in Plants. Nature, 170, (1952): 847–848.

[2] Bryant, F., and Overell, B. T.: Quantitative Chromatographic Analysis of Organic Acids in Plant Tissue Extracts. Biochem. et Biophys. Acta, 10, (1953): 471–476.

[3] Cole, H. H., and Kleiber, M.: Studies on Ruminal Gas Formation and on Consumption of Alfalfa Pasture by Cattle. J. Dai. Sci., 31, (1948): 1016–1023.

[4] Doetsch, R. N., Robinson, R. Q., Brown, R. E., and Shaw, J. C.: Catabolic Reactions of Mixed Suspensions of Bovine Rumen Bacteria. J. Dai. Sci., 36, (1953): 825–831.

[5] Ferguson, W. S., and Terry, R. A.: Bloat Investigations. J. Agric. Sci., 46, (1955): 257–266.

[6] Grau, F. H., and Halliday, W. J.: Oxidation and Decarboxylation of Citrate in the Presence of Ferrous Iron. Nature, 179, (1957): 733–734.

[7] Holton, R. W., and Noll, C. R., Jr.: A Survey of the Nonvolatile Organic Acids in Seedlings of Some Grasses and Legumes. Plant Physiol., 30, (1955): 384–386.

[8] Hungate, R. E., Fletcher, D. W., Dougherty, R. W., and Barrentine, B. F.: Microbial Activity in the Bovine Rumen: Its Measurement and Relation to Bloat. Applied Microbiol., 3, (1955): 161–173.

[9] Johns, A. T.: Bloat. Vet. Rev. & Annot., 2, (1956): 107–134.

[10] Johns, A. T.: Isolation of a Bacterium, Producing Propionic Acid, from the Rumen of Sheep. J. Gen. Microbiol., 5, (1951): 317–325.

[11] Johns, A. T.: The Mechanism of Propionic Acid Formation by *Veillonella gazogenes*. J. Gen. Microbiol., 5, (1951): 326–336.

[12] Kornberg, H. L., and Krebs, H. A.: Synthesis of Cell Constituents from C_2-Units by a Modified Tricarboxylic Acid Cycle. Nature, 179, (1957): 988–991.

[13] Lindahl, I.: The Pharmacology of Some of the Alfalfa Saponins. In "Report of the Third Conference on Rumen Function," Chicago, 1955.

[14] McBee, R. H.: Manometric Method for the Evaluation of Microbial Activity of the Rumen with Application to the Utilization of Cellulose and Hemicelluloses. Applied Microbiol., 1, (1953): 106–110.

[15] Newbold, R. P.: Preliminary Note on the Extraction of a Bloat-Promoting Fraction from Red Clover. New Zealand J. Sci. & Tech., 36, (1954): 285–286.

[16] Nichols, R. E.: A Sampling Tube for Rumen Fluid. Am. J. Vet. Res., 16, (July, 1955): 410.

[17] Ochoa, S.: Biological Mechanisms of Carboxylation and Decarboxylation. Physiol. Rev., 31, (1951): 56–106.

[18] Pierce, E. C., and Appleman, C. O.: Role of Ether-Soluble Organic Acids in the Cation-Anion Balance in Plants. Plant Physiol., 18, (1943): 224–238.

[19] Ranson, S. L.: Non-Volatile Mono-, Di-, and Tricarboxylic Acids (Chromatographic and Ion-Exchange Methods). In "Moderne Methoden der Pflanzen Analyse," vol. II. Springer-Verlag, Berlin, 1955.

[20] Scott, R. W., Burris, R. H., and Riker, A. J.: Nonvolatile Organic Acids of Crown Galls, Crown Gall Tissue Cultures and Normal Stem Tissue. Plant Physiol., 30, (1955): 355–360.

[21] Sijpesteijn, A. K., and Elsden, S. R.: The Metabolism of Succinic Acid in the Rumen of the Sheep. Biochem. J., 52, (1951): 41–45.

[22] Sirotnak, F. M., Doetsch, R. N., Robinson, R. Q., and Shaw, J. C.: Aspartate Dissimilation Reactions of Rumen Bacteria. J. Dai. Sci., 37, (1954): 531–537.

[23] Small, J.: pH and Plants. D. Van Nostrand Co., New York, N.Y., 1946.

[24] Smith, R. A., and Gunsalus, I. C.: Isocitrase: A New Tricarboxylic Acid Cleavage System. J. Am. Chem. Soc., 76, (1954): 5002.

[25] Turner, W. A., and Hartman, A. M.: The Nonvolatile Organic Acids of Alfalfa. J. Am. Chem. Soc., 47, (1925): 2044–2077.

[26] Virtanen, A. I., and Williams, F. R.: Ruminant Bloat and the Foaming Activity of Red Clover: Acta chem. scandinav., 8, (1954): 862–863.

[27] Wolfe, J. B., Ivler, D., and Rittenberg, S. C.: Malonate Decarboxylation by *Pseudomonas fluorescens*. I. Observations with Dry Cells and Cell-Free Preparations. Biol. Chem., 209, (1954): 867–873.

Summario in Interlingua

Le Etiologia de Tympania per Legumine—Acidos Nonvolatile

Extractos aquose e succos exprimite ab plantas de forrage causava un marcate evolution de gas post lor addition a specimens de contento ruminal bovin in vasos de Warburg. Le production de gas esseva plus grande in le caso de addendos preparate ab lucerna que in le caso de correspondente addendos preparate ab herba blau *(Poa pratensis)*.

Studios chimic de iste preparatos de forrage indicava que 40 a 60 pro cento de lor

potentia de producer gas esseva attribuibile a tres acidos carboxylic, i.e. le acidos citric, malonic, e succinic.

Fractiones del preparatos de forrage, obtenite per chromatographia unidimensional a papiro, indicava que le extractos plus active ab lucerna contineva acido malonic e que isto esseva absente in le extractos minus active ab herba blau. Es discutite le signification possibile de iste acidos carboxylic—e particularmente de acido malonic—in le etiologia de tympania per legumine.

Studies on Infection and Immunity with the Lungworm, *Dictyocaulus viviparus* (Bloch). I. Active Immunization of Guinea Pigs and Rabbits

A. E. WADE, Ph.D.; L. E. SWANSON, D.V.M.; LAURETTA E. FOX, Ph.D.

Gainesville, Florida

IN PREVIOUS EXPERIMENTS, it was demonstrated that guinea pigs and rabbits could be employed to study the immune responses elicited by infections with the cattle lungworm, *Dictyocaulus viviparus* (Bloch).[2,3] *Gamma*-globulin content of the serum increased and the presence of complement-fixing antibody was demonstrated following infection. It was also shown that reinfection immunity developed in these animals.[3] The present experiments were designed to determine if antigens prepared from mature lungworms, third-stage larvae, lung exudates, or lymph nodes would induce resistance to lungworm infection in guinea pigs and rabbits.

MATERIALS AND METHODS

Guinea Pig Experiments.—Thirty-five male guinea pigs, weighing 521 to 939 Gm., were subjected to cardiac puncture and 3 to 4 ml. of blood was withdrawn. They were then allotted to five groups of 7 each, depending upon their body weights, by a stratified randomization technique. By random selection, each group was assigned a particular treatment as follows: group I, larval antigen; group II, Freund's complete adjuvant; group III, lymph node antigen; group IV, buffered saline solution; and group V, whole worm antigen. The larval antigen was prepared from third-stage lungworm larvae which had been in cultures ten to 30 days prior to lyophilization. The antigen contained 331,000 larvae per milliliter of suspension, containing approximately 58,000 live larvae and 273,000 larvae which died during the culture period. Sufficient thimerosal* was added to make a concentration of 1:10,000, and the suspension was buffered in 0.9 per cent sodium chloride solution at pH 7.1. This larval antigen contained 0.873 mg. of nitrogen per milliliter as determined by micro-Kjeldahl analysis.

The lymph-node antigen was prepared from the mesenteric lymph nodes of a calf slaughtered three days following challenge with 635,000 third-stage *D. viviparus* larvae. The nodes were cleaned of fascia and ground at 5 C. in a Waring blendor for one minute with an equal weight of cold, buffered, 0.9 per cent NaCl solution. The resulting suspension was forced through gauze and the finely ground particulate material was collected. Thimerosal was added to a final concentration of 1:10,000. Micro-Kjeldahl analysis revealed that this antigen contained 6.328 mg. of total nitrogen per milliliter.

The whole-worm antigen was prepared by grinding 1.5 Gm. of lyophilized lungworms in a sterilized mortar with 13.5 ml. of 0.9 per cent sodium chloride solution buffered at pH 7.1. Merthiolate was added to make the final concentration 1:10,000. This suspension contained 5.753 mg. of total nitrogen per milliliter. All antigens were stored at −20 C. until used.

Just prior to injection, each antigen was thawed at 5 C. and emulsified with an equal volume of Freund's complete adjuvant. Isotonic sodium chloride solution, buffered at pH 7.1 and containing 0.01 per cent thimerosal, was emulsified with an equal volume of adjuvant and administered to the controls (group II). Injections of the emulsified materials were administered to each of the experimental animals at five-day intervals. The first injections of 0.4 ml. were administered intradermally, followed by two subcutaneous injections of 0.6 ml. on the fifth day and 0.8 ml. on the tenth day. On the fifteenth day, 1.0 ml. was administered intramuscularly, followed by a subcutaneous desensitizing injection of 0.1 ml. on the nineteenth day. The series of injections was completed by an intraperitoneal injection of 1.0 ml. on the 20th day. The control animals of group IV were given injections of the buffered saline solution containing 0.01 per cent thimerosal on the same days.

The challenge dose consisted of 20,000 third-stage larvae administered by stomach tube 22 days following the last injection of antigen. All animals were killed eight days (± 15 minutes) following this challenge inoculation.

The techniques of recovering the parasites from

Received for publication Jan. 25, 1960.

From the Department of Veterinary Science (Wade and Swanson) and the College of Pharmacy (Fox), University of Florida, Gainesville. Published as Florida Agricultural Experiment Station Journal Series No. 1066. Dr. Wade is now at the School of Pharmacy, University of Georgia, Athens.

Presented in part by the senior author in partial fulfillment of the requirements for the Ph.D. degree.

* Merthiolate, produced by Eli Lilly & Co., Indianapolis, Ind.

the lungs, conducting complement-fixation tests, and evaluating serum protein constituents have been described previously.[2,3]

Rabbit Experiment.—Fifteen Dutch rabbits were allotted at random to three groups of 5 each. Those in group I were given a whole worm-larva antigen; those in group II, a centrifuged lung exudate antigen; and those in group III, normal saline solution emulsified with Freund's complete adjuvant. Five milliliters of blood were withdrawn from each rabbit by cardiac puncture prior to the first inoculation with antigen. Further blood samples were obtained by incising a marginal ear vein. The whole worm-larva antigen was prepared by grinding 2.0 Gm. of mature lungworms in a TenBroeck tissue grinder with 18.0 ml. of lung exudate containing 100 larvae per milliliter. Phenol was added to make the final concentration 0.5 per cent. Just prior to the patent period of lungworm infection the lungworm exudate was collected *via* a tracheal cannula surgically inserted into the trachea of a 6-month-old calf. The exudate, collected in plastic bags attached to the cannula, was centrifuged at 3,300 g for ten minutes in an anglehead centrifuge. It was thought that this exudate might contain lungworm metabolites capable of enhancing host resistance to subsequent lungworm challenge. Since mold growth in this antigen was found in sterility tests on agar plates, 10 units of nystatin* was added per milliliter of suspension. Nystatin also was added to the exudate antigen and to the saline control. Analysis of these antigens by the micro-Kjeldahl method determined that the whole worm-larva antigen contained 2.998 mg. of nitrogen per milliliter and that the exudate antigen contained 1.506 mg. of nitrogen per milliliter, precipitable by cold, 5 per cent trichloroacetic acid.

Just prior to injection, each antigen was thawed and emulsified with an equal volume of Freund's complete adjuvant. Three injections, consisting of 2.0 ml. of the emulsion, were administered into the gluteal muscles of each rabbit at intervals of seven days. The control rabbits in group III were given normal saline solution plus 0.5 per cent phenol emulsified with an equal volume of Freund's complete adjuvant.

Twenty-five days after the third inoculation with antigen, each rabbit was given 20,000 viable third-stage larvae. Seven days following this challenge dose, the rabbits were necropsied. The lungs were immediately removed and filled with approximately 30 ml. of 0.45 per cent NaCl solution, and the lungworms were recovered as previously described.[2] After remaining in the saline solution for 18 hours at 37 C., the wire screen supporting the lung slices was removed and rinsed with a jet of fresh 0.45 per cent saline solution. This suspension was then centrifuged and the debris was suspended in 0.9 per cent NaCl solution. The residue was examined for lungworms as described previously.[2]

* Mycostatin, produced by E. R. Squibb & Sons, New York, N.Y.

RESULTS

Guinea Pig Experiments.—The injections of both antigen and saline emulsions resulted in the formation of sterile abscesses at sites of intradermal and subcutaneous injections. In 2 guinea pigs, open abscesses also appeared at the sites of the intramuscular injection of whole-worm antigen. One animal given larval antigen died 15 days after the initial injection of antigen and 2 guinea pigs given whole-worm antigen died 16 and 39 days postinjection. One animal given lymph node antigen died 25 days postinjection and 1 animal given saline solution plus Freund's adjuvant died within 24 hours after being given 20,000 lungworm larvae. Weight losses were considerable in all guinea pigs given adjuvant and were most severe following the second and fifth injections. Immediately following challenge with larvae, all guinea pigs lost weight, except those in the group given the whole-worm antigen.

The injection of both larval and whole-worm antigens resulted in the formation of complement-fixing antibodies; whereas, the injection of saline solution, saline solution plus adjuvant, or lymph node antigen produced no measurable complement-fixing antibody titers. The mean titer of terminal serums was 1:108 in guinea pigs given injections of larval antigen, and 1:42 in those given whole-worm antigen (table 1). By electrophoretic analyses of serum proteins, it was found that there were no significant quantitative differences in the components among the various groups prior

TABLE 1—Complement-Fixing Antibody Titers of Serums from Guinea Pigs Prior to the First Injection and 30 Days Following the Third Injection of Antigen

Larval antigen (group I)		Whole-worm antigen (group V)	
Preinjection titer	Terminal titer	Preinjection titer	Terminal titer
Neg.	1:32	Neg.	1:32
Neg.	1:32	Neg.	1:16
1:16	1:64	Neg.	1:64
Neg.	1:256	Neg.	1:32
Neg.	1:256	Neg.	1:64
1:32	1:8	Neg.	.....*
Neg.	.....	Neg.	.....*
Mean titers of each group			
1:7	1:108	Neg.	1:42

The serums of all animals of groups II, III, and IV were negative for complement-fixing antibody at the beginning and throughout the experimental period. The serum titer refers to the highest serum dilution in which complete inhibition of hemolysis was observed.

* Animals died during experiment.

to or following treatment. However, it was observed that a significant increase in *gamma* globulin occurred in the guinea pigs given saline solution emulsified with adjuvant and whole-worm antigen.

The guinea pigs given larval antigen harbored 50 per cent as many worms at necropsy as the controls given saline solution-adjuvant emulsion. Guinea pigs given whole-worm antigen harbored 61 per cent as many worms as did those in the control group. An analysis of variance revealed between any of the serum protein components in serum of immunized and control rabbits. It was found, however, that a significant increase in both *gamma* and *beta* globulins had occurred in all rabbits by postinjection day 21.

It was found by statistical analysis of data compiled from this experiment that a significant degree of protective immunity was produced in the rabbits given the whole worm-larva antigen. The control rabbits harbored an average of 9.2 worms, measur-

TABLE 2—The Mean Length and Numbers of Worms Recovered from the Lungs of Guinea Pigs Eight Days Following Challenge with Larvae

Group I (larval antigen)		Group II (saline solution adjuvant)		Group III (lymph node antigen)		Group IV (saline solution)		Group V (whole-worm antigen)	
No. worms	Length (μ)	No. worms	Length (μ)	No. worms	Length (μ)	No. worms	Length (μ)	No. worms	Length (μ)
164	730	392	644	392	885	407	659	1,152	727
497	809	487	748	253	724	653	812	52	806
980	677	1,544	822	703	732	363	799	1,113	802
360	783	1,183	701	272	963	352	772	117	833
257	683	590	797	1,070	766	243	769	151	861
276	715	860	602	964	790	286	815		
						183	832		
Group averages									
422	726	843	753	609	790	355	777	517	769

that these differences were not significant at the 5 per cent level. The control animals of group IV, treated with saline solution without adjuvant, harbored fewer worms than animals of any other group. This indicated that the administration of adjuvant in this quantity was detrimental to the health of the animals (table 2).

Rabbit Experiments.—The mean complement-fixation antibody titers of preinjection serums were negative for rabbits given whole worm-larva; 1.0:0.8 for those given lung exudate; and 1.0:0.8 for the controls given saline solution. Within two weeks, the serum titers had begun to increase in the principals and thereafter continued to increase in all animals until they were necropsied 46 days following the first injection of antigen.

The mean titer of the serums of rabbits given injections of whole worm-larva antigen increased to a maximum of 1:1,536. The terminal antibody titers in rabbits given lung exudate averaged 1:157 and a maximum mean titer of 1:38 was recorded in the controls (table 3).

By electrophoretic analyses, it was found that there were no significant differences ing 240 to 560 μ. The majority of these worms were sexually differentiable. The rabbits given the lung exudate antigen yielded an average of 8.0 worms, measuring 276 to 510 μ. Most of these worms were identical in size to third-stage larvae and only in 1 rabbit of this group were worms recovered that could be differentiated sexually. The rabbits given the whole worm-larva antigen yielded an average of 1.6 worms, none of which could be sexually differentiated.

DISCUSSION

The complement-fixing antibody titers in the guinea pigs given larval antigen equivalent to 1.96 mg. of nitrogen were higher than the titers of those given 12.94 mg. of nitrogen as whole-worm antigen. This may have resulted from the production of an immunologic paralysis in the animals given the whole-worm antigen. That the complement-fixing antibody titers of the serums correlate with the reduced worm burden of the guinea pigs given the larval antigen lends support to the possibility that protective antibodies were produced in response to this antigen.

TABLE 3—Complement-Fixing Antibody Titers of Serums of Rabbits Given Injections of Normal Saline Solution or Antigen Emulsified with Freund's Adjuvant

Group treatment	Preinjection	Postinjection day			
		14	21	29	46
Whole worm-larva antigen	negative	1:128	1:256	1:256	1:1,536
	negative	1:128	1:256	1:256	1:2,048
	negative	1:256	1:512	1:512	1:1,536
	negative	1:256	1:64	1:512	1:512
	negative	1:256	1:512	1:512	1:2,048
Mean titers	negative	1:205	1:320	1:410	1:1,536
	1:4	1:64	1:64	1:32	1:48
Lung-exudate antigen	negative	1:32	1:48	1:32	1:64
	negative	1:16	1:32	1:32	1:32
	negative	1:16	1:64	1:64	1:512
	negative	1:32	1:64	1:32	1:128
Mean titers	1:0.8	1:32	1:54	1:38	1:157
	negative	negative	1:16	1:16	1:32
Saline solution	negative	1:5	1:16	1:64	1:64
	negative	negative	negative	1:16	1:32
	negative	negative	1:8	1:32	1:32
	1:4	negative	1:8	1:32	1:32
Mean titers	1:0.8	1:1	1:10	1:32	1:38

Davtyan [1] reported that almost complete immunity to *Dictyocaulus filaria* infections in sheep resulted from injections of a lymph-node antigen prepared from the mesenteric lymph nodes of calves three days following challenge with *D. filaria* larvae. The negative antibody titers of animals treated with lymph-node antigen in the present study indicate that third-stage larvae were not trapped in these nodes in sufficient numbers to stimulate an immune response. It is possible that any antigen present as larvae was neutralized with antibody contained in the lymph nodes of the resistant calf from which they were removed.

TABLE 4—The Mean Length and Numbers of Worms Recovered from the Lungs of Immunized and Control Rabbits Seven Days Following Challenge with Larvae

Group I (whole worm-larva antigen)		Group II (lung-exudate antigen)		Group III (saline solution)	
Worms	Length (μ)	Worms	Length (μ)	Worms	Length (μ)
2	312	7	324	14	402
4	320	5	317	4	530
0	.....	8	326	5	468
2	316	17	393	10	560
0	.....	3	320	13	477
Group averages					
1.6	317	8	353	9.2	476

That good health is necessary for the development of protection against infection is well known. At necropsy, 2 guinea pigs given whole-worm antigen had high worm burdens, but also had sterile abscesses at the sites of intradermal, subcutaneous, and intramuscular injection. Because of their poor physical condition, they were separated from their group shortly after the second inoculation of antigen and maintained in separate cages until the termination of the experiment. This may account for the large variation in worm burdens found in this group (table 2).

Apparently, the injection of whole worm-larva antigen produced increased resistance to lungworm infection in rabbits. Not only were fewer worms recovered from the principals, but these worms were arrested in their development. Worms of smaller size were recovered from the lungs of rabbits given the lung exudate from a calf harboring immature lungworms. Complement-fixing antibodies were also present in the serums of these rabbits. These data indicate that there may be antigenic excretory or metabolic products discharged from the worms residing in the lungs and that these may stimulate the production of antibodies capable of inhibiting worm growth. They may be important in the production of immunity in calves, and may be partially responsible for the antibody which is present as early as 12 days following experimental infections with infective larvae.[4]

SUMMARY AND CONCLUSIONS

Guinea pigs given injections of lyophilized third-stage lungworm larvae emulsified with Freund's complete adjuvant harbored 50 per cent as many worms eight days following a challenge infection with

viable larvae as did the control guinea pigs given injections of isotonic sodium chloride-adjuvant emulsion. Guinea pigs given lyophilized mature lungworms emulsified with Freund's complete adjuvant harbored 61 per cent as many worms eight days following a challenge inoculation with lungworm larvae as did the control guinea pigs. Complement-fixing antibody titers of the serums of guinea pigs given injections of the larval antigen increased to 1:108, and those given whole-worm antigen increased to 1:42. Serums of guinea pigs given lymph node antigen and serums of control guinea pigs remained negative for complement-fixing antibody.

Rabbits given injections of whole worm-larva antigen (equivalent to 9.0 mg. nitrogen) emulsified with Freund's complete adjuvant harbored 17 per cent as many worms seven days following challenge with lungworm larvae as did the control rabbits given injections of normal saline solution emulsified with adjuvant. Complement-fixing antibody titers of the serums of rabbits given whole worm-larva antigen increased to a mean peak of 1:1,536, while the titer of serums from rabbits given lung exudate increased to a mean peak of 1:157 and that of the controls increased to 1:38.

The correlation between complement-fixing antibody titer and worm burdens in both guinea pigs and rabbits suggests that an antibody was formed which provided resistance to lungworm infection.

References

[1] Davtyan, E. A.: Active Immunization of Sheep Against *Dictyocaulus*. Trud. Armyan. Nauchnoissled. Vet. Inst., 8, (1955): 93–129.

[2] Wade, A. E., Fox, Lauretta E., and Swanson, L. E.: Studies on Infection and Immunity with the Cattle Lungworm, *Dictyocaulus viviparus* (Bloch). I. Infection in Laboratory Animals. Am. J. Vet. Res., 21, (Sept., 1960): 753–757.

[3] Wade, A. E., Fox, Lauretta E., and Swanson, L. E.: Studies on Infection and Immunity with the Cattle Lungworm, *Dictyocaulus viviparus* (Bloch). II. Reinfection Immunity in Guinea Pigs. Am. J. Vet. Res., 21, (Sept., 1960): 758–760.

[4] Weber, T. B.: The Complement-Fixing Antibody Response of Cattle Infected or Exposed to Reinfection with *Dictyocaulus viviparus*. Am. J. Vet. Res., 19, (April, 1958): 338–344.

SUMMARIO IN INTERLINGUA

Studios de Infection e Immunitate con le Verme de Pulmon, *Dictyocaulus viviparus* (Bloch). I. Immunisation Active de Porco de India e Conilio

Porcos de India tractate con injectiones de lyophilisate larvas de verme de pulmon del tertie stadio in emulsion con le complete adjuvante de Freund contineva—octo dies post un infection provocatori con viabile larvas—50 pro cento del numero de vermes trovate in porcos de India de controlo tractate con injectiones de isotonic chloruro de natrium in emulsion adjuvante. Porcos de India tractate con injectiones de lyophilisate vermes de pulmon matur in emulsion con le complete adjuvante de Freund contineva—octo dies post le infection provocatori con larvas de verme le pulmon—61 pro cento del numero de vermes trovate in le porcos de India de controlo. Le titros seral de anticorpore de fixation de complemento in porcos de India tractate con antigeno larval cresceva a 1:108. In porcos de India tractate con antigeno de verme matur, le titros cresceva a 1:42. Le sero de porcos de India tractate con antigeno de nodo lymphatic e le sero de porcos de India de controlo remaneva negative pro anticorpore de fixation de complemento.

Conilios tractate con antigeno de verme matur (equivalente a 9,0 mg de nitrogeno) in emulsion con le complete adjuvante de Freund contineva—septe dies post le provocation con larvas de verme de pulmon—17 pro cento del numero de vermes trovate in le conilios de controlo tractate con injectiones de normal solution salin in emulsion con adjuvante. Le titros de anticorpore de fixation de complemento in le sero de conilios recipiente antigeno de larva e verme matur cresceva al maximo medie de 1:1.536. Le titros del sero de conilios tractate con exsudato pulmonar cresceva al maximo medie de 1:157, illos del conilios de controlo a 1:38.

Le correlation inter le titros de anticorpore de fixation de complemento e le carga de vermes tanto in porcos de India como etiam in conilios suggere que un anticorpore esseva formate que provide un resistentia contra le infection per vermes de pulmon.

Studies on *Trichostrongylus axei* (Cobbold, 1879). VII. Some Quantitative and Pathologic Aspects of Natural and Experimental Infections in the Horse

S. E. LELAND, Jr., Ph.D.; J. H. DRUDGE, D.V.M., Sc.D.;
Z. N. WYANT, B.S., A.M.; G. W. ELAM

Lexington, Kentucky

A CONDITION designated by Roth and Christensen [17] as "gastritis chronica hyperplastica et erosiva circumscripta" and thought to be produced by a mechanical or toxic effect of the nematode, *Trichostrongylus axei*, occurs in the horse. Le Roux [9] described the lesions associated with *T. axei* as "ringworm-like," and Pillers [14] comments on "adenomatous-like" formations caused by this worm on the gastric mucosa.

The fund of information relating to this condition has undergone considerable development since discovery of the minute stomach worm, *T. axei*, in the mucous membrane of the equine stomach by Cobbold [1] in 1879.

Prior to the incrimination of this worm, all sorts of things had been given as the cause of this disease which also occurred enzootically in cattle.[12] The ability of *T. axei* to produce pathologic changes in the abomasum of sheep and cattle and in the stomach of the laboratory rabbit has been established with experimental infections by a number of workers.[2, 3, 6-8, 20]

Although the changes observed in the gastric mucosa of the horse have actually become pathognomonic for infection with *T. axei*, a certain amount of discussion exists in the early literature as to whether *T. axei* was responsible for the observed pathologic changes. Differences of opinion developed from information obtained at necropsy of animals naturally infected. The presence of worms with no pathologic changes in the stomach or the presence of pathologic changes and the inability to demonstrate the presence of the worm have led some authors to conclude that *T. axei* had no pathologic significance. On the other hand, numerous workers have isolated *T. axei* in the presence of inflammatory changes in the mucosa.[17]

Insofar as the present authors are aware, no record exists of the experimental production of these pathologic changes in the horse.

The purposes of the present study were (1) to experimentally produce the pathologic changes of this condition by infection with *T. axei*, and (2) to observe certain quantitative aspects of the infection, such as prepatent period, male/female worm ratio, egg-laying potential of the worm, and the influence of duration of infection. In addition, observations for comparison of the pathologic manifestations of natural and experimental infections were made.

MATERIALS AND METHODS

Animals and Animal Facilities.—Horses used for the experimental infections were selected on the basis of no *T. axei* larvae in three or more seven-day-old fecal cultures subjected to the Baerman technique. All 3 animals of this group were Thoroughbreds. Horse 1 was a yearling and horses 2 and 3 were weanlings. Experimentally infected animals were kept in box stalls and not allowed access to pasture. Routine manure removal was maintained throughout the study. The animals were provided with grain and hay free from domestic animal fecal contamination.

Horses 8 (Thoroughbred mare), 618 (crossbred yearling), 619 (crossbred yearling), E-113 (12-year-old Thoroughbred mare), and E-21 (19-year-old Thoroughbred mare) harbored naturally acquired infections of *T. axei*. The existence of infection was ascertained by identification of the characteristic larvae in seven-day-old fecal cultures subjected to the Baerman technique. Horses 618 and 619 were also part of an anthelmintic evaluation and had been given 358 mg./kg. and 215 mg./kg. of piperazine citrate, respectively, seven days prior to necropsy. However, daily pre- and post-treatment egg and larval counts did not indicate any drug activity against *T. axei*.

Infection Technique.—The strain of *T. axei* used in the experimental infections was originally ob-

Received for publication March 7, 1960.

From the Department of Animal Pathology, University of Kentucky, Lexington.

This investigation is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the director of the Station.

The work was supported in part by a grant from the Grayson Foundation, Inc.

tained from a horse in Kentucky (strain A)[7, 8] and was maintained in helminth-free lambs, calves, and rabbits for approximately one year before being used in the present experiments. In a three-year period, during which time this strain of the worm was alternated between sheep, calves, rabbits, and horses, no change in infectivity was detected.[8-9] In horse 1, the larvae, counted by the dilution method, were mixed with a half gallon of oats and were readily consumed. Larvae were administered to horses 2 and 3 with the aid of a stomach tube. Several rinses of the container and tube were made to insure the total deposition of larvae within the stomach.

Details of larval cultivation, egg counts, and worm recovery methods, including the artificial digestion technique, were essentially the same as recorded in earlier papers[8-9] with the exception that worm counts were based on numbers of worms recovered from the stomach wall digest, stomach contents, and first 6 to 8 inches of the duodenum.

The serosal and smooth muscle layers were removed from the stomachs, which reduced the amount of artificial digestive juice required and facilitated spreading the stomach specimens for photographing. Since morphologically similar eggs from worm species other than *T. axei* were present in the 24-hour fecal collections, the egg-laying potential of the female worm was evaluated in terms of the characteristic *T. axei* larvae recovered from 100-Gm., seven-day-old fecal cultures.

All tissue sections were stained with hematoxylin and eosin.

RESULTS

Experimental Infections.—No pathologic manifestations were observed in horse 1, which had been given 6,800 larvae and from which only 18 adult worms were recovered at necropsy 46 days after inoculation.

From horse 2, given 1,500,000 larvae, and horse 3, given 1,862,000 larvae, 176,099 and 74,400 adult worms were recovered, respectively. In these 2 animals, marked alterations in the gastric mucosa were apparent. Horse 2 was necropsied 56 days after inoculation; *i.e.*, after administration of the infective third stage larvae. This animal gained 56 lb. during the 56 days of infection (1 lb./day). In the fundus gland region of the stomach (fig. 1), an area 6 by 3 inches was observed which was composed of circumscribed areas of catarrhal inflammation. These circumscribed areas were surrounded by an area of hyperemia.

Horse 3 was necropsied 395 days after inoculation. This animal gained only 130 lb. during 13 months of infection (0.32 lb./day). The fundic and pyloric regions of the stomach and the first few inches of the duodenum had extensive pathologic manifestations (fig. 2). The fundic region contained numerous white, raised, circumscribed plaque-like areas which became progressively more numerous and contiguous toward the pyloric region. The surface of the plaques were covered with a heavy mucous exudate containing numerous *T. axei*. However, greater numbers of worms were observed at the periphery of the lesions. In the distal portion of the pyloric region, the circumscribed areas were more elevated and more nearly resembled nodules. In the duodenum were two polyps (fig. 2, 5) which measured 1.5 cm. in diameter and 1.5 cm. in elevation. Worms were present in histologic tissue sections (fig. 7) from several areas of the polyp. The polyp was covered by hyperplastic epithelium and was supported to some extent by connective

TABLE 1—Worm Counts and Related Data Pertaining to Trichostrongylus axei Infection in Horses

Horse No.	Approx. age	No. larvae given	Days from infection to necropsy	Prepatent period (days)	Worms Recovered					
					From stomach contents	From stomach digest	Total	In relation to larvae administered (%)	Male/ female	Larvae/ female/ 24 hr.
1	Yearling*	6,800	46	27	.....	18	18	0.3	0.66	.....
2	Weanling*	1,500,000	56	24	53,232	122,867	176,099	11.7	0.52	.....
3	Weanling*	1,862,000	395	25	0	74,400	74,400	4.0	0.51	.....
8	Mare	.....	.....	....	.....	.....	35,500	.....	0.91	.....
618	Yearling	.....	.....	....	.....	.....	56,100	.....	0.95	32.2
619	Yearling	.....	.....	....	.....	.....	116,200	.....	0.75	12.1
E-21	Mare	.....	.....	....	.....	.....	.....	.....	.....	.....
E-113	Mare	.....	.....	....	.....	.....	.....	.....	.....	.....
Averages				25.3					0.72	22.1

* Age of horses experimentally infected; other horses were naturally infected, age given at time of necropsy.

tissue. Increased alveolation or honeycombing was evident at the dorsal portion of the polyp (fig. 5). The lymphocyte was the principal infiltrating cell, and large numbers of these cells were present throughout the polyp. Occasionally, areas with large numbers of eosinophils were noticed. Pinpoint areas of calcification were present in the mucosa. Excessive mucous production and necrosis were evident in many areas (fig. 6).

Microscopic examination of lesions observed in the stomach of horse 3 revealed pathologic changes essentially the same as those observed in the natural infections reported by the present and previous authors.[17] The surface epithelium in some areas was eroded to the extent that the gastric glands were exposed, and other areas of the same stomach had extensive hyperplasia of the surface epithelium. In either case, the areas were usually covered with a thick layer of mucus, cells, cellular debris, and necrotic material which formed a diphtheritic-type membrane (fig. 14). Only occasionally, however, did the membrane survive the histologic sectioning procedure. Hypertrophy of the gastric glands was observed. Lymphocytes were the principal cell infiltrating the lamina propria, and large numbers were observed both in the vicinity of worm sections and in areas where worms were not observed but pathologic changes existed. Occasionally, large numbers of eosinophils were noticed. Hyperemia was prevalent. Increased interstitial connective tissue with streaks of ramifying smooth musculature was observed.

Natural Infections.—Only slight changes were observed in naturally infected horse 8 and consisted of circumscribed areas of hyperemia on the gastric mucosa.

Lesions in the stomachs of naturally infected horses 618 and 619 were similar in type and extent. The lesions (fig. 3) were located in the fundus and appeared as whitish, slightly raised, circumscribed areas which were surrounded by hyperemic areas. Except for being somewhat more clearly demarcated and occupying a slightly greater area, the lesions in the stomachs of these 2 naturally infected animals were comparable with the gross lesions observed in the stomach of experimentally infected horse 2.

Microscopic examination of the stomach lesions of horses 618 and 619 revealed large numbers of worm sections on the epithelial surface, in the gastric pits, and occasionally in the gastric glands. The lamina propria about the gastric pits contained large numbers of lymphocytes. Involvement of the deeper layers of the mucosa, *i.e.*, the gastric glands and associated connective tissue, was slight at this stage of the infection.

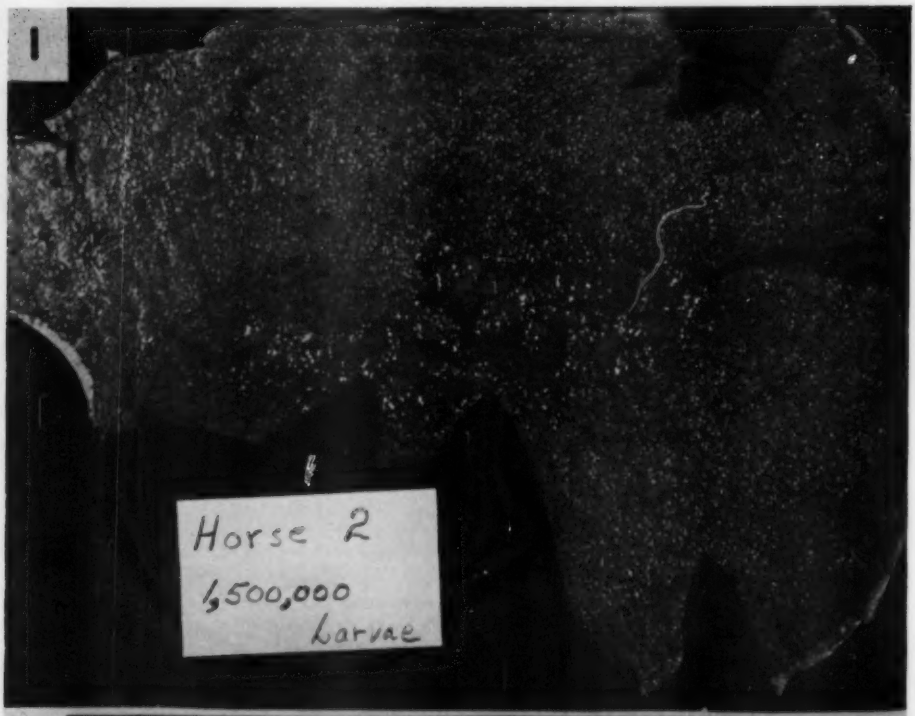
Horse E-21 was a 19-year-old, barren mare which was brought to the department for destruction. Stomach lesions were largely confined to and completely occupied the fundus and consisted of approximately 60 circumscribed pathologic formations. Thirty-five of the lesions were nodules measuring 1 to 2 cm. in diameter and ½ to 1 cm. in elevation ((fig. 4). The remaining 25 lesions differed in that they were ulcers with depressed rather than raised centers (fig. 4). The nodules were covered with the white mucous layer observed on the stomach lesions of horses 2, 3, 618, and 619. In general, the ulcers appeared as denuded forms of the nodules. The areas between the nodules or ulcers were hyperemic.

A cross-section of one of the nodules is shown (fig. 9). Microscopic examination revealed hyperplasia of the gastric epithelium, hypertrophy of the gastric glands, increased alveolation with cuboidal cell lining, extensive hyperemia, and mucus production. Muscular strands were observed penetrating the lamina propria perpendicular to the muscularis mucosae. An increase in the interstitial connective tissue, which contained many infiltrating lympho-

Legends for Illustrations on Opposite Page

Fig. 1—Horse 2, with experimental infection of 56 days duration, had circumscribed areas of catarrhal inflammation in fundus gland region of the stomach. The circumscribed areas were surrounded by areas of hyperemia; x 0.3.

Fig. 2—Horse 3, with experimental infection of 395 days duration, had the plaque-like areas in the fundus region of the stomach which become progressively more numerous toward the pyloric region. Two polyps were present in the duodenum; x 0.3.


1
Horse 2
1,500,000
Larvae

2
HORSE #3
1,860,000
T. AXEI LARVAE

cytes and some eosinophils, was particularly noticeable. Numerous worm sections were observed on the epithelial surface and imbedded in the alveolar cavities (fig. 10), gastric pits, and glands. Occasionally, these alveolar cavities became encapsulated with connective tissue and infiltrating cells. Contents of the cavities were similar to the material of the diphtheritic-type membrane but, in addition, were calcified (fig. 13).

On microscopic examination of sections of the stomach ulcers, considerable necrosis and sloughing of the mucous membrane was seen (fig. 11 and 12). In some areas, the mucous membrane had been reduced to less than half the normal thickness which exposed the gastric glands.

The lesions of naturally infected horse E-21, although somewhat more extensive and perhaps of longer duration, were comparable to the lesions of experimentally infected horse 3.

Horse E-113, a 12-year-old mare that died suddenly, was given to the department for necropsy. Peritonitis of some duration was present. A ruptured diaphragm and a 6-inch rupture in the fundus region of the stomach were observed. Examination of the gastric mucosa revealed typical lesions of *T. axei* infection throughout the fundic portion of the stomach. Numerous worms of this species were readily recovered from these lesions. Also present in this animal were an exceptionally small aneurysm of the anterior mesenteric artery and a moderate to light bot infection (approximately 100 second- and third-instar *Gastrophilus intestinalis*). Although no statement of finality can be made with respect to the precise cause of the stomach rupture, the associated pathologic changes produced by *T. axei* in the fundus were undoubtedly a contributing factor, if not the primary cause.

Data pertaining to worm counts and related information are presented (table 1).

Worm recoveries ranged from 18 to 176,099. The percentage developments (adult worms recovered/larvae given) of the 3 experimentally infected animals were: horse 1, 0.3 per cent; horse 2, 11.7 per cent, and horse 3, 4.0 per cent. The male/female worm ratios for 6 animals ranged from 0.51 to 0.95, with an average of 0.72 (72 males per 100 females). Worm larvae *(T. axei)* per female worm per 24 hours in horses 618 and 619 were 32.2 and 12.1, respectively, with an average of 22.1.

DISCUSSION

According to the results of the present experimental infections, infection with *T. axei* can bring about, directly or indirectly, characteristic pathologic changes in the gastroduodenal mucosa of the horse. Changes observed in the experimentally infected animals were essentially the same as those observed in the natural infections in the present study and in natural infections reported by other authors.[17]

Two of the 3 experimentally infected horses developed lesions following inoculation. In the third animal, a low-grade infection was established and undoubtedly accounted for the lack of pathologic manifestations. The small number of larvae administered and the method of larval administration (on grain) in this animal are considered responsible for the low level of infection.

The pathologic manifestations appear to be a function of at least two factors, *i.e.*, the worm burden and the duration of infection.

Experience with pure infections of this worm in lambs [8] and calves [7] also has shown that low-grade infections are not accompanied by significant pathologic changes. In the present work, slight pathologic changes were observed in the presence of 35,500 worms at necropsy, and, at 56,100 worms, characteristic lesions of *T. axei* infection were present. However, these worm counts were from natural infections where the exact exposure (number of infective larvae) or the duration of infection were not known (other than the fact that horse 618 was less than 2 years old). Therefore, the number of worms necessary to initiate the observed changes may have been greater or smaller than recovered at the time of necropsy.

The level of infection encountered in horse 8 (35,500 worms at necropsy) was apparently sufficient to produce the observed circumscribed areas of hyperemia of the gastric mucosa. This type of mild manifestation probably also represents the earliest gross pathology associated with animals destined (those having heavier worm burdens) for more extensive involvement.

Experimentally infected horse 2 showed stomach lesions comparable to naturally infected horses 618, 619, and E-113 and consisted of hyperemia, various degrees of lymphocytic and catarrhal inflammation,

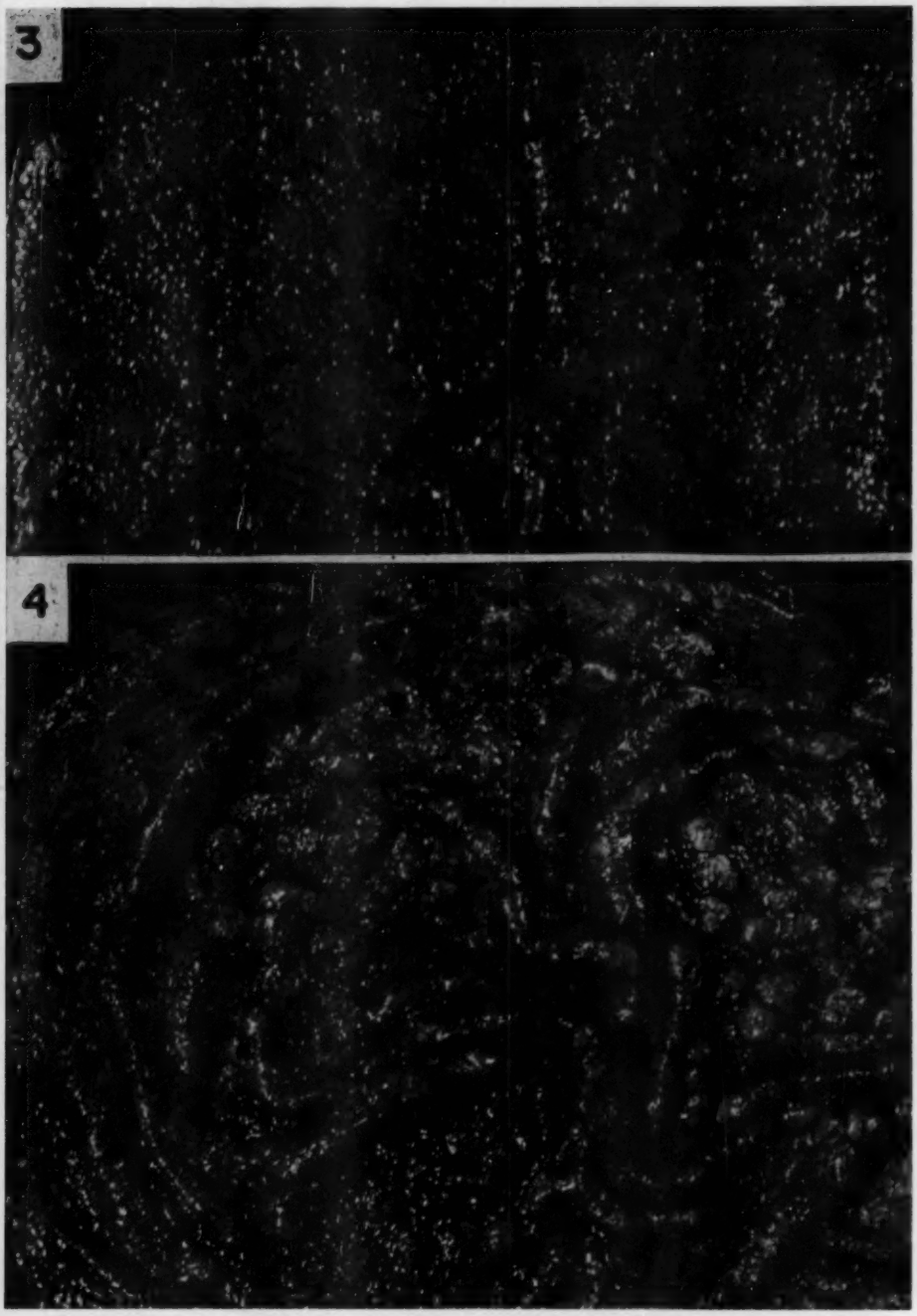


Fig. 3—Horse 619, natural infection, fundus region, which was comparable to the experimental infection shown in figure 1; x 0.7.

Fig. 4—Mare E-21 (19 years old), natural infection, fundus region, showing nodules and ulcers. Approximately x 0.4.

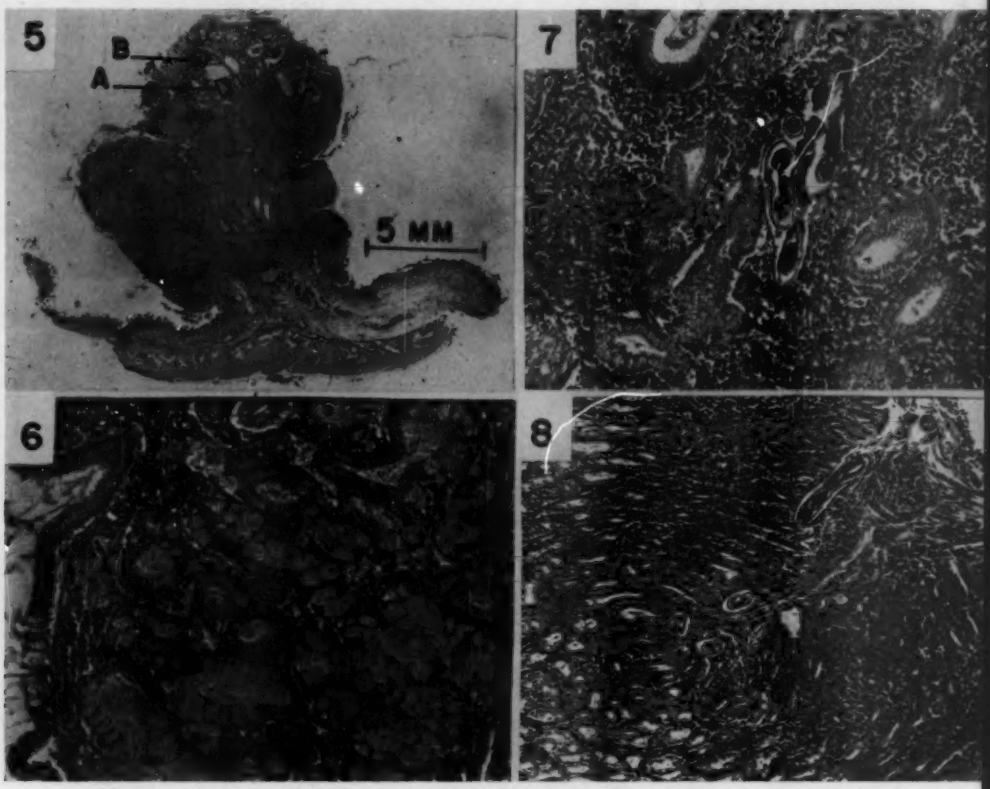


Fig. 5—Experimental infection. Cross-section of a duodenal polyp shown in figure 2, showing alveolation and the extent of proliferation.

Fig. 6—Higher magnification of area A in figure 5 showing excessive mucus production and necrosis. Approximately x 140.

Fig. 7—Higher magnification of area B in figure 5 showing a coiled worm and the cellular infiltration. Approximately x 140.

Fig. 8—Stomach section of experimental infection (horse 3), showing worm in a gastric pit with associated cellular infiltration; x 60.

necrosis, and erosion of the mucous membrane. In the case of horse 2, this stage of the lesion was produced in 56 days.

Experimentally infected horse 3, although not as extensive or perhaps as advanced, had stomach lesions comparable to those of naturally infected horse E-21. In these animals, hyperplastic and hypertrophic changes of the gastric mucosa were apparent in addition to the changes listed above. Duration of infection in horse 3, however, was 395 days. Horse E-21 was 19 years old so that, in this case, the duration of infection could have been somewhat longer and the re-exposure extensive.

The developmental pathology of the stomach lesion appears to begin with a hyperemia which, depending upon a number of factors, progresses to lymphocytic and catarrhal inflammation, necrosis, erosion of the mucous epithelium and, thence, to chronic proliferative inflammation. All stages may be present in the same stomach. The proliferative changes, however, were the result of a rather long duration of infection since they were not observed 56 days after inoculation and had progressed only moderately (when compared with natural infections) 395 days after inoculation.

To what extent, if at all, a vitamin A

deficiency was a factor in the observed pathologic manifestations was not within the scope of the present experimental design. Vitamin A deficiency occurs in the horse, and stall-confined animals would be particularly susceptible. In the present study, the experimental animals were provided with corn, oats, and a good quality of hay, which fulfilled the daily vitamin A requirements. None of the signs of severe vitamin A deficiency, such as night blindness, uneven or poor hoof development, degeneration of nerves, or increased susceptibility to respiratory diseases, were observed in any of the horses of the present study. Furthermore, numerous horses have been used throughout the years in this department under conditions similar to those of the present study and have not developed a detectable vitamin A deficiency, nor have the pathologic changes associated with *T. axei* infection been observed in the absence of the worm. The 2 heavily infected experimental animals (horses 2 and 3) consistently consumed their daily ration. This is in contrast to results of experiments with

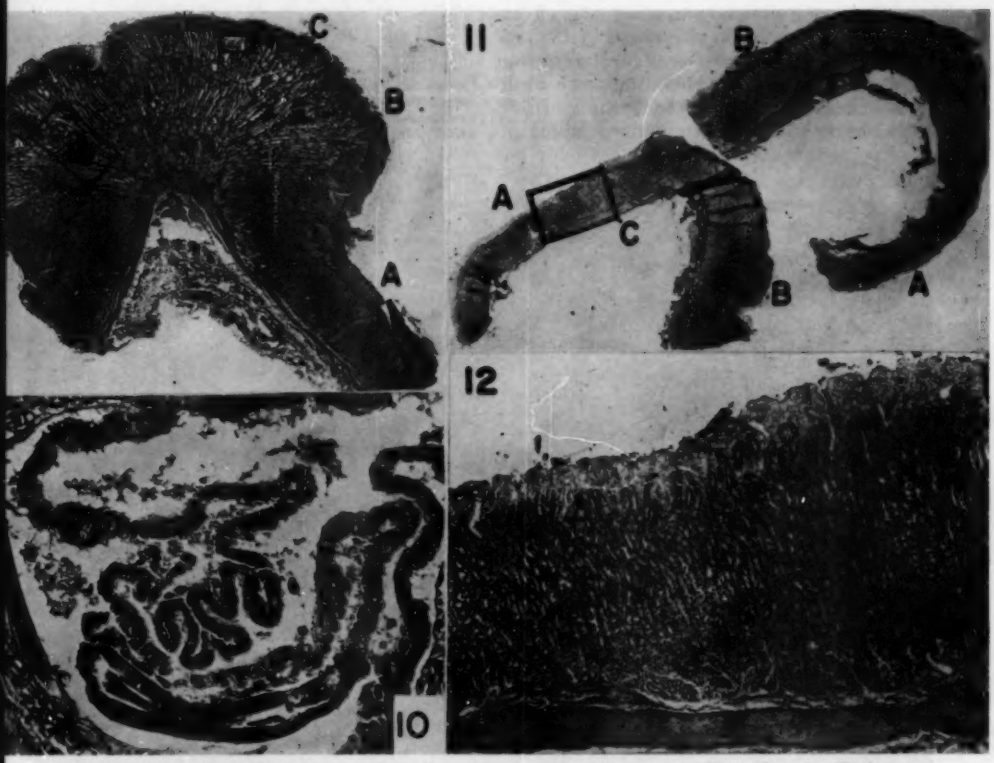


Fig. 9—Natural infection, cross-section of a stomach nodule from mare E-21 (fig. 4). Notice the approximately normal thickness of the mucosa at point A in comparison to the proliferative thickening at B. Note diphtheritic-type membrane at A. Increased alveolation is apparent between the two points. One of the alveolar spaces filled with cells and debris has become encapsulated at point D (see enlargement, fig. 13). The muscularis mucosae is beginning ramification of the overlying highly proliferated mucosa; x 6.

Fig. 10—Higher magnification of area C in figure 9 showing worm coiled just beneath surface epithelium of an alveolar cavity; x 170.

Fig. 11—Natural infection, cross-sections of stomach ulcers from mare E-21, figure 4. Notice reduced thickness between center of ulcer at points A and edge at points B; x 4.

Fig. 12—Higher magnification of area C in figure 11 showing diphtheritic-like membrane, necrosis, and reduced thickness of the mucosa. Approximately x 30.

sheep.[8] The rate of gain in these 2 animals was, however, markedly reduced.

In the forestomach of rats, the prominent features of a vitamin A deficiency are "blunt epithelial down growths," elevated plaques with central ulcerations, papillomas, edema of the submucosa connective tissue, inflammatory infiltrate in the edematous layers, and mitotic figures in areas of active hyperplasia.[5] Similarities in the pathologic changes between the vitamin A-deficient rat and the *T. axei*-infected horse obviously exist. Hitchcock and Bell [5] consider *Gongylonema neoplasticum* (a nematode parasite of the rat forestomach) to be an effective irritant enhancing the effects of vitamin A deficiency upon the squamous forestomach and state, as a corollary, that vitamin A deprivation heightens the pathologic changes attributable to the worm.

Thus, the relationship between avitaminosis A and *T. axei* infection is not clearly defined and requires further study.*

The observations of the present study are compatible with the theory that the disease-producing potential of *T. axei* is not primarily related to the mechanical irritation of the worm itself but to a secretion (toxin) or excretion produced by the worm.[4, 10] Lymphocytic inflammation of mucous membranes, which usually develops into catarrhal or chronic proliferative inflammation, has been associated with toxic and some slightly virulent infectious agents acting over a period of time.[18] Proliferation of simple epithelium and the under-

* As this report goes to press, experiments concerning this relationship have just been completed. Rats which are naturally refractive to *T. axei* and which were rendered visibly vitamin A-deficient were still refractive to the worm. It appears that factors other than, or in addition to, vitamin A deficiency govern at least susceptibility to this worm.

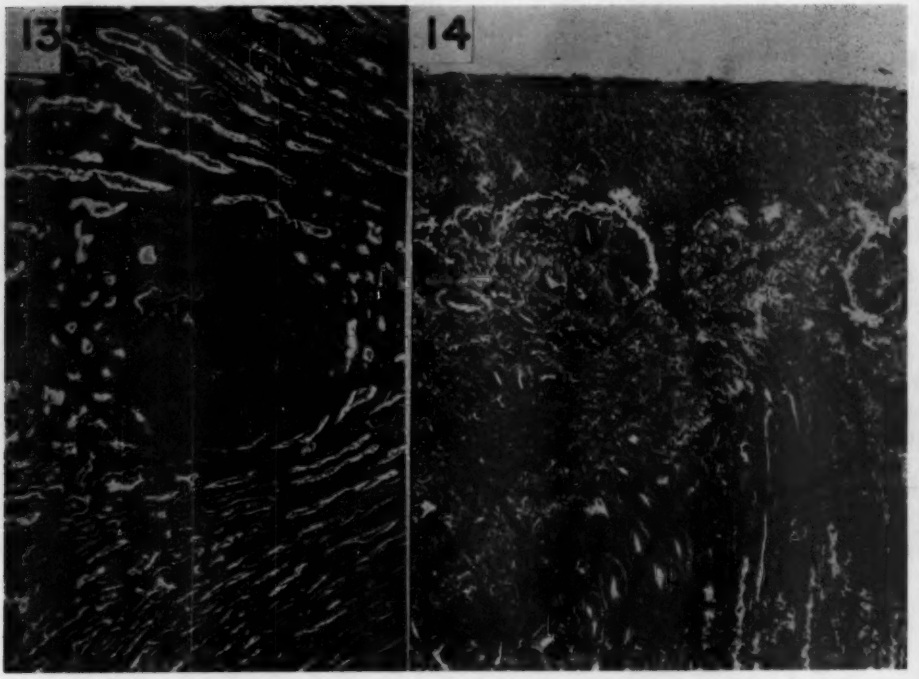


Fig. 13—Higher magnification of encapsulated alveolar cavity D shown in figure 9. Notice intact wall of alveolar cavity. The cavity itself was filled with material similar to that of the diphtheritic-like membrane; x 25.

Fig. 14—Experimental infection, horse 3, section through one of the plaque-like areas in the fundic region of the stomach shown in figure 2. Note diphtheritic-type membrane and necrosis of underlying tissue; x 76.

lying stroma with the production of multiple papillary projections are regarded as a response to continued mechanical or chemical irritations.[18]

The observations of the present study conform to the foregoing pattern of inflammatory changes and suggests the existence of an irritant acting over a long period. Whether the irritant was the worm itself, a metabolite of the worm, or a product of tissue destruction cannot be determined with finality from existing information. In any event, the irritant was sufficiently potent to produce these inflammatory changes as well as actual necrosis and sloughing of the mucosa.

Although the precise functional significance of the lymphocyte is not known, a number of the suggested functions appear compatible with the present findings. The circulating as well as the fixed lymphocytes are under the control of the adrenal steroid hormones. When the level of these hormones increases under a stress situation produced by tissue injury, the lymphocytes congregated in the area of injury are lysed and thought by some to release a high concentration of antibody into the vital focus.[16] It has also been suggested that lymphocytes are concerned with handling foreign protein that enters the tissues undigested.[15] They are regarded by others as concerned in the absorption and neutralization of toxins.[11]

The excessive numbers of eosinophils infiltrating the gastric mucosa, although not as numerous as the lymphocytes, are in keeping with the association of this cell with helminth infections.[19] Because eosinophils also are associated with allergic disorders,[19] it is tempting to suspect that a sensitization to the worm or its products may have developed. The prolonged duration of infection possible with this worm (known from unpublished experiments by the authors to be as long as 4 years in rabbits following a single inoculation of a pure culture) would allow ample exposure to existing sensitinogens. However, an important role in detoxification has also been attributable to the eosinophil.[19]

Worthy of mention at this point is the fact that age immunity against *T. axei* in the horse does not appear to be a well-established entity. This is attested by the severe natural infections observed in the 12- and 19-year-old mares of the present study. In addition, in these animals, which were undoubtedly subjected to more or less continuous re-exposure to the worm, the opportunity for sensitization appears great.

The average prepatent period of 3 horses was 25.3 days and compares with 21.3 days in calves,[7] 23.3 days in lambs,[8] and 22.7 days in rabbits.[6] The same strain of *T. axei* was used in all cases. The male/female worm ratio averaged 0.72 in 6 horses and compares with 0.77 in calves,[7] 0.57 in sheep,[8] and 0.82 in rabbits.[6]

Since morphologically similar eggs from worm species other than *T. axei* were present in the 24-hour fecal collections from the horse, the egg-laying potential of the female worm was evaluated in terms of the characteristic *T. axei* larvae recovered from 100-Gm., seven-day-old fecal cultures. Due to mortality and losses in the cultivation and recovery manipulations, larval counts usually are less than the corresponding worm-egg counts derived from the same fecal sample. Thus, an average of 22.1 *T. axei* larvae per adult female *T. axei* per 24 hours was observed in 2 horses and compares with averages of 41.6 and 20.3 eggs passed per 24 hours per female worm in pure infection studies of calves[7] and rabbits,[6] respectively.

SUMMARY

Horses experimentally infected with *Trichostrongylus axei* were compared with naturally infected animals with respect to pathologic manifestations and quantitative aspects of the infection.

The typical pathologic changes of the gastroduodenal mucosa observed in association with natural infections of *T. axei* in the horse were produced experimentally by inoculation of horses with this worm. Koch's postulate, requiring the experimental production of the disease in a susceptible animal by the parasite, was thus fulfilled.

The developmental pathology of the stomach lesion begins with a hyperemia which, depending upon a number of factors, progresses to lymphocytic and catarrhal inflammation, necrosis, erosion of the mucous epithelium and thence to chronic proliferative inflammation. All stages may be present in the same stomach. The proliferative changes, however, were the result of a rather long duration of infection because they had progressed only moderately at 395 days after inoculation.

An average prepatent period of 25.3 days in 3 horses and an average male/female worm ratio of 0.72 in 6 horses were observed. An average ratio of 22.1 *T. axei* larvae per adult female *T. axei* per 24 hours was observed in 2 horses.

References

[1] Cobbold, T. S.: Parasites; A Treatise on the Entozoa of Man and Animals; Including Some Account of the Entozoa. London, (1879): 383.

[2] Doran, D.: The Course of Infection and Pathogenic Effect of *Trichostrongylus axei* in Calves. Am. J. Vet. Res., 16, (July, 1955): 401–409.

[3] Gibson, T. E.: The Pathogenesis of *Trichostrongylus axei* in Sheep. State Vet. J., Great Britain, (Sept. 25, 1951): 1–6.

[4] Gibson, T. E.: Studies on Trichostrongylosis. I. The Pathogenesis of *Trichostrongylus axei* in Sheep Maintained on a Low Plane of Nutrition. J. Comp. Path. & Therap., 64, (1954): 127–140.

[5] Hitchcock, C. R., and Bell, E. T.: Studies on the Nematode Parasite *Gongylonema neoplasticum*, and Avitaminosis A in the Forestomach of Rats: Comparison with Fibiger's Results. J. Nat. Cancer Inst., 12, (1952): 1345–1387.

[6] Leland, S. E., Jr., and Drudge, J. H.: Studies on *Trichostrongylus axei* (Cobbold, 1879). II. Some Quantitative Aspects of Experimental Infection in Rabbits. J. Parasitol., 43, (1957): 160–166.

[7] Leland, S. E., Jr., Drudge, J. H., Wyant, Z. N., Elam, G. W., and Hutzler, L. B.: Studies on *Trichostrongylus axei* (Cobbold, 1879). IV. Some Aspects of Treatment, Pathogenicity, and Quantification in Experimental Infections of a Horse Strain in Calves. Am. J. Vet. Res., 20, (Sept., 1959): 787–794.

[8] Leland, S. E., Jr., Drudge, J. H., Wyant, Z. N., and Elam, G. W.: Studies on *Trichostrongylus axei* (Cobbold, 1879). V. Some Quantitative and Pathologic Aspects of Experimental Infections with a Horse Strain in Sheep. Am. J. Vet. Res., 21, (May, 1960): 449–457.

[9] Le Roux, P. L.: Helminths Collected from Equines in Edinburgh and in London. J. Helminthol., 2, (1924): 111–134.

[10] Morgan, B. B., and Hawkins, P. A.: Veterinary Helminthology. Burgess Publishing Co., Minneapolis, Minn., 1949.

[11] Ogilvie, R. F.: Pathological Histology. 5th ed. Williams & Wilkins Co., Baltimore, Md., 1957.

[12] Pappenheimer, A. M., and Larimore, L. D.: The Occurrence of Gastric Lesions in Rats: Their Relation to Dietary Deficiency and Hair Ingestion. J. Exptl. Med., 40, (1924): 719–732.

[13] Penberthy, J.: Parasitic Gastro-Enteritis, Diarrhoea, and Anemia of Cattle. J. Comp. Path. & Therap., 7, (1894): 249–263.

[14] Pillers, A. W. N.: Parasites and Parasitic Diseases. A System of Veterinary Medicine (by various authors). Edited by Wallis Hoare, London, 2, (1915): 1405–1566.

[15] Rich, A. R., Lewis, M. R., and Wentrobe, M. M.: The Activity of the Lymphocyte in the Body's Reaction to Foreign Protein, as Established by the Identification of the Acute Splenic Tumor Cell. Johns Hopkins Hosp. Bull., Baltimore, Md., 65, (1939): 311–327.

[16] Robbins, S. L.: Textbook of Pathology with Clinical Applications. W. B. Saunders Co., Philadelphia, Pa., 1957.

[17] Roth, H., and Christensen, N. O.: On Parasitic Gastritis in the Horse Due to *Trichostrongylus axei*. Skand. Vet.-tidskr., 32, (1942): 488–514.

[18] Smith, H. A., and Jones, T. C.: Veterinary Pathology. Lea & Febiger, Philadelphia, Pa., 1957.

[19] Strong, R. P.: Stitt's Diagnosis, Prevention, and Treatment of Tropical Diseases. Vol. II, 7th ed. Blakiston Co., Philadelphia, Pa., (1945).

[20] Turner, J. H., and Kates, K. C.: The Pathogenic Effect on Lambs of a Bovine Strain of the Stomach Hairworm, *Trichostrongylus axei*. J. Parasitol., 40, Sec. 2, (1954): 13.

SUMMARIO IN INTERLINGUA

Studios in *Trichostrongylus axei* (Cobbold, 1879). VII. Aspectos Quantitative e Pathologic de Infectiones Natural e Experimental in le Cavallo

Cavallos experimentalmente inficite con *Trichostrongylus axei* esseva comparate con naturalmente inficite cavallos con respecto al manifestationes pathologic e al aspectos quantitative del infection.

Le typic alterationes pathologic del tunica mucose gastroduodenal que esseva observate in infectiones natural con *T. axei* occurreva etiam in cavallos subjicite a inoculation experimental del verme. Le postulato de Koch, demandante le production experimental del morbo per le parasito in un susceptibile animal, esseva assi satisfacite.

Le pathologia disveloppamental del lesiones gastric comencia in un hyperemia le qual—in dependentia de un numero de factores—progrede verso inflammation lymphocytic e catarrhal, necrose, erosion del epithelio mucose, e postea a chronic inflammation proliferative. Omne le mentionate stadios pote esser representate in le mesme stomacho. Tamen, le alterationes proliferative esseva le resultato de un satis longe periodo de activitate. Illos habeva progredite solmente a grados moderate 395 dies post le inoculation.

Un periodo prepatente medie de 25,3 dies in 3 cavallos e un proportion mascule-feminin medie del vermes de 0,72 in 6 cavallos esseva observate. Un production medie de 22,1 larvas de *T. axei* per adulte verme feminin per 24 horas esseva constatate in 2 cavallos.

Recovery of Western Equine Encephalomyelitis Virus from Crop Washings of Experimentally Infected Pigeons

JOHN F. WINN, D.V.M., M.P.H., and DAN F. PALMER, B.S., M.P.H.

Atlanta, Georgia

IN A PREVIOUS STUDY,[4] it was found that pigeons are susceptible to at least 160 chick $l.d._{50}$ of western equine encephalitis (WEE) virus, given subcutaneously, and that virus could be recovered from blood 24 to 96 hours postinoculation. Since 1 uninoculated bird in the control group became infected, possibly by contact with his cage mates or through environmental contamination produced by droppings of infected birds, this phase was investigated. The possibility that virus might be present in crop secretion also was investigated.

MATERIALS AND METHODS

Before inoculation, 5 young adult pigeons had neutralizing indexes of less than 8 against WEE, as shown by serum-neutralization test. They were housed in individual cages of hardware cloth, with catch pans for collection of fecal specimens. Each bird was given 104 chick $l.d._{50}$ of WEE virus intradermally. Feces were collected five times a day, pooled in a 50-ml. centrifuge bottle, and kept at 4 C. At the end of each day, the sample was emulsified with an equal amount of 20 per cent, inactivated, normal horse serum and centrifuged at 9,230 g for 20 minutes in a Servall SS4 angle-head centrifuge. After 4 ml. of supernatant fluid was removed, 1 ml. of antibiotic solution, containing 2,500 units of penicillin, 1 mg. of dihydrostreptomycin, and 5 mg. of sodium sulfadiazine, was added to inhibit bacterial growth. After incubation at 4 C. overnight, 0.3 ml. of each separate sample was injected subcutaneously into 4 chicks[1]; 0.5 ml. of each sample was plated on blood agar, and 0.5 ml. of each was inoculated into two tubes containing 5 ml. of thioglycollate broth each. The plates and broth were observed for seven days. One tube of thioglycollate was incubated at room temperature, 22 to 25 C.; the other tube and blood plate were incubated at 37 C.

Portions of brains from chicks dying within 48 hours postinoculation were harvested and cultured as described. Verification of virus isolates was made by the U.S. Public Health Service, Communicable Disease Center, Montgomery, Ala.

Samples of oral secretions and crop effluvium were collected on sterile absorbent cotton swabs by wiping out the faucial areas of throats of the pigeons. Each swab was washed in 5 ml. of 20 per cent, inactivated, normal horse serum. The suspension was centrifuged at 9,230 g for 20 minutes, and 1 ml. of the antibiotic solution added to 4 ml. of the supernatant fluid. The specimen was then treated, as were the fecal specimens. Hydrogen ion concentrations of suspensions and throat washings were determined with a Beckman Model G pH meter. Additional determinations were made on feces suspended in distilled water and on crop swabs washed in distilled water.

Blood specimens were collected every six hours for one day following inoculation, then once a day for five days. Blood dilutions to determine virus concentrations were done as described previously.

RESULTS

One of the 5 birds died due to dyserasia which kept the blood from clotting after the preinoculation blood sample was taken. Virus was not recovered from any of 24 fecal specimens collected over a five-day period. The average pH of feces suspended in distilled water was 5.75. When the diluent was 20 per cent horse serum, as used in the inoculum, the average pH was 6.6.

TABLE 1—Titer in Logs of Western Equine Encephalomyelitis Virus in Pigeon Blood Following Inoculation of 104 Chick $l.d._{50}$

Pigeon No.	No. of hours postinoculation							
	6	12	18	24	48	72	96	120
P1	<1.0	1.5	2.5	6.0	5.5	3.7	<3.5	<1.0
P2	<1.0	<1.0	2.5	5.7	5.7	3.5	1.3	<1.0
P3	<1.0	<1.0	2.5	6.3	5.7	3.5	2.7	<1.0
P4	<1.0	2.7	<3.5	6.3	6.0	3.5	1.5	<1.0

The oral and crop washings from 3 of 4 of the remaining pigeons contained demonstrable WEE virus. Virus was present in both the 48-hour and 72-hour specimens from 2 of these pigeons, but in only the 48-hour crop washing from the other bird. Virus was not recovered from the crop after 96 hours, even though WEE virus was still present in the blood of all 4 birds. The average pH of raw crop washings was 6.4; after mixing with the 20 per cent horse serum, the average pH was 7.2.

From the U.S. Public Health Service, Communicable Disease Center, Atlanta, Ga.

Virus in blood specimens was first found 12 hours postinoculation. The l.d.$_{50}$ titers of virus for each of the pigeons are summarized (table 1). Virus was not recovered 120 hours after inoculation.

Material inoculated onto blood agar and thioglycollate was bacteriologically negative during the time of and at the temperature of incubation.

DISCUSSION

During the first 3½ to 5 weeks of life, young pigeons are fed on regurgitated crop contents (called "pigeon milk") from the parent birds. Inasmuch as WEE virus was found in the crop washings, there is a possibility that young pigeons become infected via this route, although the susceptibility of young pigeons to infection by feeding virus has not been established. Some virus infections are known to occur via the gut in mammals,[5,6] even when this is not considered the usual route of infection. During the studies of Reeves *et al.*[7] on the latency of WEE virus, virus was recovered from birds several months after inoculation, indicating that there are many complexities in the "overwintering" of this disease. Thus, we have a possibility that young birds could be infected from the parent bird, could serve as a source of virus for mosquitoes, and could become infected by mosquitoes. These results seem to confirm the findings of other investigators that an acid environment is inimical to encephalitis virus.[2,3,8] The alkaline inoculum contained virus which was viable and demonstrable; the acid inoculum contained no demonstrable virus. It seems reasonable to assume that, if the virus were present in the crop, it would pass through the gut and be excreted. Of course, there are many factors which might denature the virus, such as digestive juice and the acid environment in the cloaca. The findings indicated that 20 per cent horse serum did not buffer feces to the alkaline range, but it did change the pH of the crop washings enough to permit recovery of virus.

The presence of virus in the blood indicated that pigeons can be infected by the oral route with 104 chick l.d.$_{50}$ and that viremia appears as early as 12 hours postinoculation. The ultimate growth of virus was in the same concentration as reported previously,[4,9] and virus fell below detectable levels at the same time as reported.

SUMMARY

It was thought important to investigate the possibility that western equine encephalomyelitis (WEE) virus might be present in the excrement and crop secretions of experimentally infected pigeons. Five adult pigeons, having neutralizing indexes of less than 8 against WEE, were inoculated with 104 chick l.d.$_{50}$ of WEE virus. One bird died due to a blood dyscrasia. Virus was present in the crop secretions from 3 of 4 of the remaining pigeons, and in all of the blood samples taken 18 to 96 hours postinoculation. Virus was not recovered from any of the fecal specimens.

References

[1] Chamberlain, R. W., Sikes, R. K., and Kissling, R. E.: Use of Chicks in Eastern and Western Encephalitis Studies. J. Immunol., 73, (1954): 106–114.

[2] Duffy, C. E.: pH Stability of St. Louis Encephalitis Virus. Proc. Soc. Exptl. Biol. & Med., 63, (1946): 333–334.

[3] Finkelstein, H., Marx, W., Beard, D., and Beard, J. W.: pH Stability of Equine Encephalomyelitis (Eastern Strain) Under Various Conditions. J. Infect. Dis., 66, (1940): 117–126.

[4] Kaplan, W., Winn, J. F., and Palmer, D. F.: Susceptibility of the Pigeon (Columbia livia) to Infection with Western Equine Encephalomyelitis Virus. J. Immunol., 75, (1955): 225–226.

[5] Reagan, R. L., Straud, N., and Brueckner, A. L.: Response of the Swiss Albino Mouse to Semliki Forest Virus. Cornell Vet., 43, (1953): 427–430.

[6] Reagan, R. L., Yancey, F. S., Chang, S. C., and Brueckner, A. L.: Studies of the Sindbis Virus. I. Susceptibility of the Cave Bat by Various Routes of Exposure. II. Susceptibility of the Suckling Hamster by Various Routes of Exposure. Am. J. Trop. Med. & Hyg., 5, (1956): 1032–1035.

[7] Reeves, W. C., Hutson, G. A., Bellamy, R. E., and Scrivani, R. P.: Chronic Latent Infection of Bird with Western Equine Encephalomyelitis Virus. Proc. Soc. Exptl. Biol. & Med., 97, (1958): 733–736.

[8] Sharp, D. G.: Purification and Properties of Animal Viruses. Adv. Virus Res., 1, (1953): 277–313.

[9] Winn, J. F., Palmer, D. F., and Kaplan, W.: Development and Persistence of Western Equine Encephalitis Virus Antibodies in Experimentally Infected Pigeons. Cornell Vet., 47, (1957): 337–343.

SUMMARIO IN INTERLINGUA

Virus de Encephalomyelitis Equin Occidental, Retrovate in Elutiones Ingluvial de Experimentalmente Inficite Columbas

Esseva considerate como importante investigar le possibilitate que virus de encephalomyelitis equin occidental (EEO) es presente in le excrementos e le secretiones ingluvial de experimentalmente inficite columbas. Cinque columbas adulte, con indices neutralisante

contra EEO de minus que 8, recipeva inoculationes de virus de EEO in un concentration amontante a 104 DL_{50} de galletto. Un del aves moriva in consequentia de un dyscrasia de sanguine. Virus esseva presente in le secretiones ingluvial de 3 del 4 remanente columbas e in omne le specimens de sanguine obtenite inter 18 e 96 horas post le inoculation. Nulle virus esseva retrovate in ulle del specimens fecal studiate.

The Position of the Canine Prostate Gland

NORMA GORDON, M.A., B.V.Sc., M.V.Sc., M.R.C.V.S.

Guelph, Ontario

IN RECENT YEARS, the topographic anatomy of the canine prostate gland has assumed importance as a result of new techniques for the surgical removal of the gland [2,11,14] and because of the increased use of roentgenograms in the diagnosis of prostatic disorders.[1,16,18] In view of these developments, the existing descriptions have proved inadequate; the present investigation was conducted to meet the needs of the clinician, radiologist, and surgeon.

A review of the literature reveals a wide divergence of opinion as to the normal position of the canine prostate gland; no correlation between the age of the dog and the position of the prostate has been attempted and none of the authors state the number of dogs examined. Leisering,[12] Ellenberger and Baum,[8] Bradley,[6] Matera,[14] and Schnelle [18] consider the prostate to be mainly an abdominal organ. Lamy,[11] Bourdelle and Bressou,[5] Dobberstein and Koch,[7] and Getty [9] either state or illustrate it to be a pelvic organ; whereas, Miller,[15] Sisson and Grossman,[21] and Bloom [4] state that the position of the prostate in relation to the pelvic brim is influenced by the fullness of the bladder and the distension of the rectum.

MATERIALS AND METHODS

A total of 140 dogs (newborn to 19 years old) and eight 52-day fetuses were examined. The age was known accurately for about 80 per cent of the dogs and was estimated for the remainder from the general appearance, the body condition, the teeth, and, in the dissected dogs, by the presence or absence of the thymus.[3] Using one of the following three methods, the position of the prostate was determined in relation to the cranial borders of the pubes, which delimit the pelvis from the abdomen ventrally. This landmark can be readily located by palpation or radiography.

I. *Gross Dissection.*—Seventy-three dogs were examined by gross dissection.

II. *Roentgenography.*—Thirty dogs and 8 fetuses were studied by taking lateral-exposure roentgenographs using special techniques. The lateral-exposure roentgenogram is considered to be the best method of outlining the abnormal prostate but an unsatisfactory method of outlining the normal gland.[1,16,18] However, it was found that, if special techniques using radio-opaque substances or contrast mediums were employed, any part of the gland that was in the abdominal cavity became visible and, if the location of the gland was pelvic or partly pelvic, its approximate position could be judged from the position of the terminal parts of the ductus deferens.

The special techniques were (a) pneumoperitoneum; *i.e.*, injection of air into the peritoneal cavity; (b) cystourethrography; *i.e.*, injection of contrast mediums into the bladder and urethra; (c) double-contrast cystourethrography; *i.e.*, the injection of contrast mediums followed by air into the bladder and urethra; (d) the injection of contrast mediums into the ductus deferens; (e) a combination of two or three of these techniques.

The relationship between the posture of the dog and the position of the prostate was investigated by taking lateral cystourethrograms of the animal in three positions: (1) in a normal standing position with the spine horizontal, (2) with the forelimbs raised and the spine vertical, and (3) with the hindlimbs raised and the spine vertical. To determine the influence on the position of the prostate of the fullness of the bladder and the distension of the rectum, or both, lateral cystourethrograms were taken of the dog in a normal standing position, with the bladder full, then empty, with the rectum full, then empty, and with both the bladder and rectum full, then empty.

Each dog was killed by an overdose of pentobarbital sodium after roentgenography, and, if pneumoperitoneum had been employed, the air was aspirated from the peritoneal cavity. In all cases, a 10 per cent solution of gelatin colored by the addition of India ink was then injected into the peritoneal cavity. In some cases, the bladder was filled with a 10 per cent gelatin solution of a contrasting color. The dog was frozen for three days in exactly the same position as for roentgenography, and serial sagittal sections were cut with a bandsaw to verify the roentgenologic findings.

III. *Rectal Palpation.*—Rectal palpation was used to determine the position of the prostate in

Received for publication May 11, 1959.

From the Department of Anatomy, Ontario Veterinary College, Guelph, Ont., Canada. Miss Gordon is now with the Sub-Department of Veterinary Anatomy, University of Cambridge, England.

Taken from a thesis submitted to the University of Toronto in partial fulfillment of the requirements of the M.V.Sc. degree.

The author acknowledges the advice and encouragement of Dr. J. H. Ballantyne and Dr. W. C. D. Hare, and the help of Dr. R. J. Chorley, Department of Geography, University of Cambridge, with the graph.

37 dogs and, in some of these, the effect of posture and bladder distension were examined.

Calculation of the Means.—It is assumed that gross dissection and lateral-exposure roentgenography are equally reliable and that rectal palpation is only two thirds as reliable as the other two methods. To allow for this, a weighting factor equal to the reliability is applied to the data; *i.e.*, the number of cases in which the prostate gland is found in a given position by method III is taken to be two thirds of the number shown in table 1, and for methods I and II the number is taken to be the number shown.

The lines in figure 1 represent the weighted mean position in any age group. This may be described mathematically as follows: If W is the reliability factor for a given method and n the number of observations found by the method to have a position x abdominal ($x = 0$, 1/3, 2/3, or 1). The points are plotted at $\frac{\Sigma nWx}{\Sigma nW}$ where the summations are taken of all three methods and all the positions.

Results

The positions of the prostate glands in the dogs examined are illustrated (fig. 1, table 1), with the exception of 3 aged dogs which were either castrated or cryptorchid and whose prostates were atypically pelvic in position. The smooth curve in figure 1 relates the weighted mean positions of the prostate in the six age groups to the age of the dogs in each group; figure 1 thus graphically represents only generalized findings.

In eight 52-day fetuses, 7 newborn dogs, and 2 pups less than 2 months old, the prostate was entirely abdominal (fig. 1, start of phase A) due to the attachment of the bladder to the umbilicus by the urachus

TABLE 1—Position of the Prostate Gland in 140 Dogs and Eight Fetuses

AGE	METHOD	PELVIC	1/3 ABDOMINAL	2/3 ABDOMINAL	ABDOMINAL	TOTAL	LIFE STAGE
<2 months	I	—	—	—	2	2	
	II	—	—	—	15	15	
	III	—	—	—	—	—	URACHUS RUPTURE
2-8 months	I	9	—	—	—	9	
	II	3	—	—	—	3	
	III	5	—	—	—	5	SEXUAL MATURITY
8-12 months	I	—	6	—	—	6	
	II	—	—	—	—	—	
	III	5	6	—	1	12	
1-2 YEARS	I	3	10	1	—	14	
	II	—	—	—	—	—	
	III	—	—	—	—	—	
2-5 YEARS	I	—	—	9	2	11	
	II	—	—	5	—	5	
	III	1	6	—	—	7	
>5 YEARS	I	—	—	10	21	31	
	II	—	—	8	7	15	
	III	1	1	7	4	13	OLDEST DOG 19 YEARS

Method I, gross dissection; II, lateral exposure roentgenogram; and III, rectal palpation. The position of the gland was assessed by reference to the cranial borders of the pubes which delimits the pelvis from the abdomen ventrally.

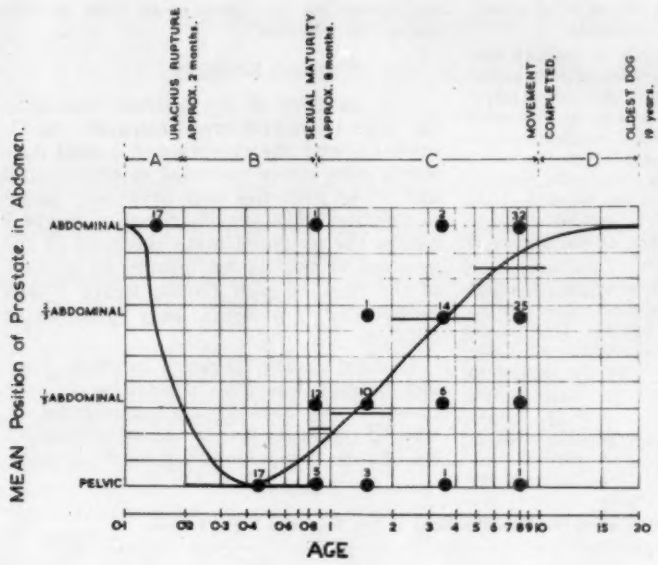


Fig. 1—Generalized relationship between the position of the canine prostate and the age of the dog. The position of the dots indicates age and classification; the number which accompanies each dot represents the number of dogs. Lines represent the weighted mean positions in any age group and are drawn to cover the complete age groups.

(fig. 2). The urachus gradually degenerates after birth and the loss of this attachment frees the cranial end of the bladder which moves caudad through the abdominal cavity, with the result that the prostate changes in position from abdominal to pelvic (fig. 1, later part of phase A). In the 17 pups between 2 and 8 months old (fig. 1, phase B), the prostate was entirely pelvic in position, still being small and immature (fig. 3). This group is considered to span the age period between the rupture of the urachus and the beginning of sexual maturity. The position of the gland in dogs 8 months to approximately 10 years old (fig. 4, 5) and its gradual change from a pelvic to an abdominal organ is shown (fig. 1, phase C).

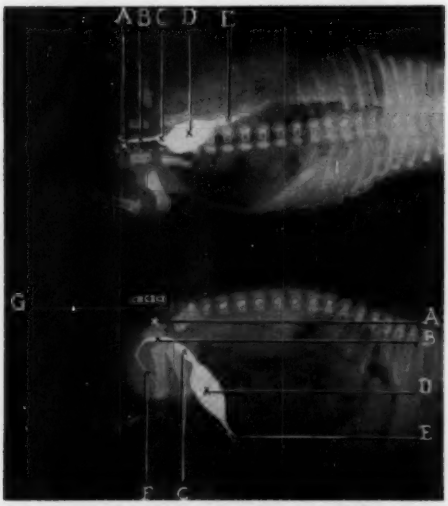


Fig. 2—Ventrodorsal and lateral cystourethrograms with pneumoperitoneum in a 52-day fetus injected with contrast medium. (A) fossa pararectalis, (B) urethra pelvina, (C) urethra prostatica, (D) vesica urinaria, and (E) urachus.

After sexual maturity, at approximately 8 months of age, there is a relative increase in the size of the prostate due to androgenic stimulation, and the change in its position occurs concurrently with this increase in size (fig. 4).

In dogs 2 to 5 years old (fig. 5), the prostate is approximately two thirds abdominal in position. It is of interest that the present investigation confirmed a progressive increase in the size of the gland during this period, which bears on the observation of Schlotthauer and Bolman [20] that there is a constant relationship between the weight of the prostate and the body weight of the animal. In all of the dogs over 5 years old (fig. 6), the prostate

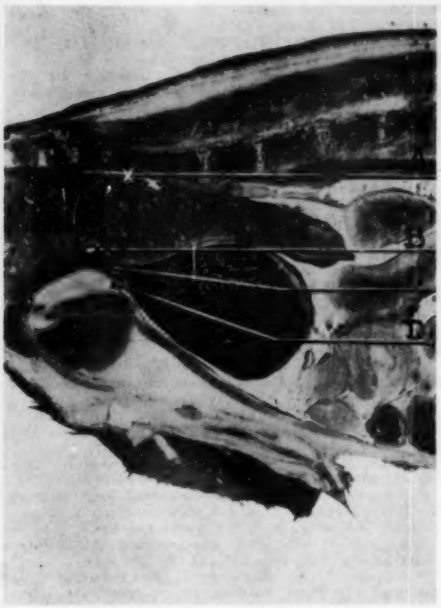


Fig. 3—Midsagittal section of a 6-month-old dog with the bladder and urethra filled with black gelatin and the peritoneal cavity filled with white gelatin. (A) fossa pararectalis, (B) fossa paravesicalis, (C) prostata, and (D) fossa rectovesicalis.

was enlarged, confirming previous observations,[10, 19, 20, 22] and it is noteworthy that the gland in dogs of this age group was found to be in an abdominal position. The position of the prostate was not affected by distension of the bladder or rectum, nor did postural factors have any effect in old

Fig. 4—Cystourethrogram of the ductus deferens of a 9-month-old dog injected with contrast medium. (A) ductus deferens and (B) vesica urinaria. The contrast medium mixed completely with the urine and outlines the bladder.

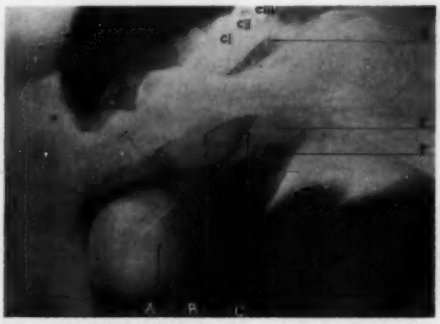


Fig. 5—Cystourethrogram of a 2-year-old dog with pneumoperitoneum and the ductus deferens injected with contrast medium. (A) vesica urinaria, (B) rectum, (C) ductus deferens, (D) fossa pararectalis, (E) prostata, and (F) fossa paravesicalis.

dogs. In young dogs, however, where the prostate was small, it was found to drop slightly caudad in the pelvis when the forelimbs were raised and the spine was vertical.

Conclusions

It is considered that there was a relationship between the age of the dog and the

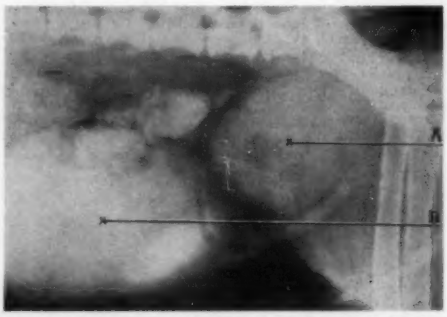


Fig. 6—Lateral roentgenogram of a 12-year-old dog with a greatly enlarged prostate. (A) prostata; (B) vesica urinaria.

position of the prostate, which should enable the clinician and radiologist to predict the position of the gland in any normal dog whose age can be determined.

In young pups where the urachus is still present and holds the bladder forward, the prostate is abdominal in position. After the rupture of the urachus, the bladder no longer draws the prostate craniad and it becomes a pelvic organ. After sexual maturity, the prostate becomes progressively

more abdominal in position with the increasing age of the dog.

Other physical factors were not found to influence the positions of the prostate to any marked extent.

SUMMARY

The position of the prostate gland was measured in 140 dogs up to 19 years old, and eight 52-day fetuses. They were examined by means of gross dissection, roentgenography with gross serial sections, and rectal palpation.

A relationship was found between the position of the prostate, measured in relation to the brim of the pelvis, and the age of the dog.

In animals which were prenatal, newborn, and less than 2 months old, the gland was abdominal in position; whereas, in pups 2 to 8 months old, the gland was pelvic in position. After sexual maturity, the prostate increased in size and, with increasing age, changed its position from pelvic to abdominal. After the age of about 10 years, the prostate can be considered as an abdominal organ.

References

[1] Archibald, J., and Bishop, E. J.: Radiographic Visualization of the Canine Prostate Gland. J.A.V.M.A., 128, (April 1, 1956): 337–342.

[2] Archibald, J., and Cawley, A. J.: Canine Prostectomy. J.A.V.M.A., 128, (Feb. 15, 1956): 173–177.

[3] Baum, H.: Die Thymusdrüse des Hundes. Deutsche Ztschr. f. Thiermed., 17, (1890–1891): 349–354.

[4] Bloom, F.: Pathology of the Dog and Cat. American Veterinary Publications, Inc., Evanston, Ill. (1954).

[5] Bourdelle, E., and Bressou, C.: Anatomie Régionale des Animaux Domestiques. IV. Chien et Chat. J. B. Ballière et Fils, Paris, (1953).

[6] Bradley, O. C.: Topographical Anatomy of the Dog. 5th ed. Oliver & Boyd, London, (1948).

[7] Dobberstein, J., and Koch, T.: Lehrbuch der vergleichenden Anatomie der Haustiere. S. Hirzel, Leipzig, (1954).

[8] Ellenberger, W., and Baum, H.: Anatomie des Hundes. Paul Parey, Berlin, (1891).

[9] Getty, R.: Atlas for Applied Veterinary Anatomy. Burgess Publishing Co., Minneapolis, (1955).

[10] Kracht-Paléjeff. Arch. f. wissensch. prakt. Thierh., 37, (1910–11): 299. (cited by Zuckermann, S., and Groome, J. R.: The Aetiology of Benign Enlargement of the Prostate in the Dog. J. Path. Bact., 44, [1937]: 113–124.)

[11] Lamy, E.: Anatomie et Chirugie de la Prostate du Chien. Thésis, l'École Vétérinaire d'Alfort. Vigot Frères, Paris, (1931).

[12] Leisering, A. G. T.: Atlas der Anatomie des Pferdes und der übrigen Haustiere. 2nd ed. B. G. Teubner, Leipzig. (1888).

[13] Lord, L. H., Rowsell, H. C., Cowley, A. J., and Archibald, J.: Suppurative Protatitis Corrected by Prostatectomy. N. Amer. Vet., 37, (1956): 963–967.

[14] Matera, E. A.: Prostatectomia pela via perineal no cao. Thesis, Foeuldade de Medacina Veterinaria da Universidade de São Paulo, Brazil, (1952).

[15] Miller, M. E.: Guide to the Dissection of the Dog. 3rd ed. Edwards Brothers, Inc., Ann Arbor, Mich.

[16] Pommer, A.: Die Röntgendiagnostik und Therapie der Prostataerkrankungen des Hundes. Berl. u. Münch. tierärztl. Wchnschr., 22–23, (1956): 453–457.

[17] Schnelle, G. B.: The Bladder and the Prostate Gland. North Am. Vet., 21, (1940): 222–227.

[18] Schnelle, G. B.: Radiology in Small Animal Practice. 2nd ed. North American Veterinary Publications, Evanston, Ill., (1950).

[19] Schlotthauer, C. F.: Observations on the Prostate Gland of the Dog. J.A.V.M.A., 81, (Nov., 1932): 645–650.

[20] Schlotthauer, C. F., and Bolman, J. L.: The Prostate Gland of the Dog. Cornell Vet., 26, (1936): 343–349.

[21] Sisson, S., and Grossman, J. D.: The Anatomy of the Domestic Animals. 4th ed. W. B. Saunders Co., Philadelphia, (1953).

[22] Smith, L. W.: Senile Changes of the Testes and Prostate in Dogs. J. Med. Res., 40, (1919): 31–51.

[23] Zuckermann, S., and McKeown, T.: The Canine Prostate in Relation to Normal and Abnormal Testicular Changes. J. Path. & Bact., 46, (1938): 7–19.

SUMMARIO IN INTERLINGUA

Le Position del Prostata Canin

Le position del prostata esseva mesurate in 140 canes de etates de usque a 19 annos e in octo fetos de 52 dies. Le examine esseva executate per dissection macroscopic, per roentgenographia con sectiones macroscopic in serie, e per palpation rectal.

Esseva constatate un relation inter le position del prostata, mesurate con respecto al margine del pelve, e le etate del can.

In animales que esseva prenatal o neonate e de etates de minus que 2 menses, le position del glandula esseva abdominal. In juvenes de etates de inter 2 e 8 menses, le position del glandula esseva pelvic. Post le attingimento del maturitate sexual, le prostata accresceva su dimensiones e—con le avantiamento del etate—alterava su position ab pelvic ad abdominal. Al etate de approximativemente 10 annos, le prostata pote esser considerate como un organo abdominal.

Use of Emulsified Fat for Intraperitoneal Alimentation

M. J. BURNS, Ph.D., and C. H. CLARK, D.V.M., Ph.D.

Auburn, Alabama

THE PROBLEM of supplying sufficient calories to the malnourished patient is an important one. The obvious solution is to supply the caloric requirements largely as emulsified fats. Such preparations provide a concentrated source of energy in a form that is non-irritating to mucous membranes.

An abundance of work has been done concerning the intravenous use of emulsified fat and has been thoroughly reviewed by many authors.[1] When emulsified fat is given intravenously, it should be given slowly; consequently, a considerable amount of the clinician's time is required. Also, venipuncture is often difficult in malnourished patients.

A review of parenteral nutrition has been presented by Geyer.[2] Glucose and protein hydrolysates have been used intraperitoneally in veterinary medicine for several years. No reference was found concerning the use of fat intraperitoneally. The objective of this experiment was to measure the rate of absorption of emulsified fat from the peritoneal cavity and to determine the effect of intraperitoneal injections on rats.

MATERIALS AND METHODS

The albino rats used in the experiments weighed 120 to 290 Gm. and were of both sexes. They were caged individually and given food and water ad libitum.

The emulsified fat used in this work contained 15 per cent cottonseed oil, 4 per cent dextrose, and sufficient phosphatides to disperse the fat.* It was prepared by high-pressure homogenation, and the particle size was less than 1 μ in diameter. This product has been used extensively in man but has always been administered intravenously. Each milliliter provides about 1.6 calories.

Received for publication Feb. 15, 1960.

From the Department of Physiology and Pharmacology, School of Veterinary Medicine, Auburn University, Ala., where Dr. Burns is associate professor and Dr. Clark is head professor. Approved as Paper No. 858, Publications Committee.

In these studies, the Raolein was supplied by Abbott Laboratories and the Lipomul was supplied by the Upjohn Company.

* Lipomul I.V., produced by the Upjohn Company, Kalamazoo, Mich.

The emulsified fat was tagged by mixing it with I^{131}-labeled triolein* in a blender so that the mixture gave about 25,000 counts of radioactivity per milliliter per minute. The mixture was very uniform in radioactivity after standing for 24 hours at 38 C.

The amount of radioactivity remaining in the peritoneal cavity was used as a measure of the emulsified fat not absorbed. To remove the fat from the peritoneal cavity a ventral midline incision was made into the peritoneal cavity and the fluid remaining in the cavity was drained into a beaker; the cavity was then washed thoroughly with distilled water and the washings were added to the beaker. The total volume was measured and the amount of radioactivity in three 3.0-ml. samples was determined with a scintillation counter and medical spectrometer. The average of the three counts was used to determine the total amount of radioactivity remaining in the peritoneal cavity.

Experiment 1.—To determine the rate of absorption of emulsified fat from the peritoneal cavity, 80 rats were given intraperitoneally either 5 ml. or 10 ml. of tagged emulsified fat. The rats given 5-ml. injections had an average weight of 164 Gm.; those given 10-ml. injections had an average weight of 238 Gm. Consequently, the rats given 5-ml. injections received an average of 3.1 ml. per 100 Gm. of body weight, while those given 10-ml. injections received 4.2 ml. per 100 Gm. of body weight. Thus, the dose did not differ greatly when expressed on a body weight basis.

Four rats given 5-ml. injections and 4 given 10-ml. injections were killed 0, 0.5, 1, 2, 4, 8, 16, 32, 64, and 72 hours after the initial injections. Thus, a total of 80 rats were killed and the amount of emulsified fat remaining in the peritoneal cavity was determined as described.

Experiment 2.—The total absorption of three different levels of tagged emulsified fat was determined in this experiment. Intraperitoneally, 5 rats were given 2.5 ml. of fat per 100 Gm. of body weight, 5 were given 5.0 ml., and 5 others were given 7.5 ml. The rats were killed 24 hours later and the amount of radioactive fat remaining in the peritoneal cavity was determined as described.

Experiment 3.—This experiment was conducted to determine the effect of daily intraperitoneal injections of untagged emulsified fat on the health

* Raolein, produced by Abbott Laboratories, North Chicago, Ill.

of rats. They were given daily injections of 2.5, 5.0, or 7.5 ml. per 100 Gm. of body weight. The rats weighed 120 to 200 Gm. Five rats were used for each dosage level of fat. Necropsies were performed on the rats at the termination of the experiment.

RESULTS

The results of the first experiment, concerning the rate of absorption of the tagged emulsified fat, are shown (fig. 1). There was little difference in the per cent of uptake of the 5.0-ml. and 10.0-ml. injections; however, larger rats were given the 10.0-ml. doses. The rate of absorption was rapid at first, then decreased rapidly after about 80 per cent of the fat had been absorbed. About 20 hours were required for the absorption of 80 per cent of the fat.

The results of the second experiment, which concerned the total absorption after 24 hours for the various levels of fat injected, are shown (table 1). An average of

TABLE 1—Absorption of Emulsified Fat From Peritoneal Cavity after 24 Hours

Item	Fat injected per 100 Gm. body weight		
	2.5 ml.	5.0 ml.	7.5 ml.
Per cent absorbed	83.8	62.0	44.6
Total (Gm.) absorbed per 100 Gm. body weight	2.1	3.1	3.4
Total (Gm.) not absorbed per 100 Gm. body weight	0.4	1.9	4.1

Five rats were used per group.

83.8 per cent at the 2.5 ml./100 Gm. rate was absorbed after 24 hours. Only 62 per cent at the 5.0-ml. dose rate and 44.6 per cent at the 7.5-ml. dose rate were absorbed. The total quantity absorbed was 2.1 Gm., 3.1 Gm., and 3.4 Gm. for the 2.5-, 5.0-, and 7.5-ml. doses, respectively.

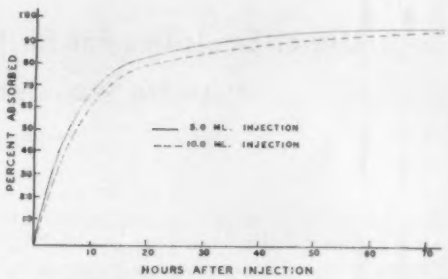


Fig. 1—Absorption of radioactive fat from the peritoneal cavity. Each curve represents data from 40 rats killed in groups of 4 at varying intervals.

The effect of the daily intraperitoneal injections of the various levels of emulsified fat as described in the third experiment is shown (fig. 2). All of the rats given more than 2.5 ml. of fat daily per 100 Gm. of body weight developed severe ascites after five to ten days. None of the rats given only 2.5 ml. of fat per 100 Gm. of body weight had developed ascites after three weeks of treatment.

Fibrin-like material and multiple organized adhesions were found in the peritoneal cavities of rats in addition to the ascitic fluid. Cystic masses containing emulsified fat were found also. In rats given tagged emulsified fat, the fibrin-like material was found to possess considerable radioactivity. No abnormalities were observed in the rats that did not develop ascites.

DISCUSSION

About 2.5 ml. of emulsified fat per 100 Gm. of body weight appears to be the maximum that can be tolerated by the rat. The oil appeared to separate from the emulsion and a fibrin-like material formed in the animals that were given the high

Fig. 2—Rats given daily injections of emulsified fat for 14 days. (A) control, (B) 2.5 ml./100 Gm. of body weight, (C) 5.0 ml./100 Gm. of body weight, and (D) 7.5 ml./100 Gm. of body weight. Notice severe ascites in rats C and D.

levels of fat. The fibrin-like material did not accumulate in the animals that were given the low level of fat. The ascites that resulted from intraperitoneal administration of emulsified fat was probably caused by accumulation of fat and fibrin-like material in the intraperitoneal cavity. The fibrin-like masses and adhesions probably caused a mechanical obstruction of lymphatic vessels which resulted in accumulation of additional fat and other fluids.

About 4 calories of energy would be supplied by 2.5 ml. of emulsified fat. This amount is less than half of the calories needed by a 100-Gm. rat for daily maintenance; consequently, less than half of the caloric requirement of the rat could be provided by intraperitoneal alimentation.

Summary and Conclusions

1) The absorption of emulsified fat from the peritoneal cavity has been demonstrated in the rat. Three to 4 ml. per 100 Gm. of body weight was the maximum amount of emulsified fat that could be absorbed by a normal rat in 24 hours.

2) The daily administration of as little as 5 ml. of emulsified fat per 100 Gm. of body weight caused severe ascites in rats after five to ten days. None of the rats given 2.5 ml. per 100 Gm. of body weight developed ascites.

References

[1] Anon.: Symposium on Intravenous Fat Emulsions. Metabolism, 6, (1957): 591–831.

[2] Geyer, Robert P.: Parenteral Nutrition. Physiol. Rev., 40, (1960): 150–186.

Summario in Interlingua

Le Uso de Grassia Emulsificate in le Alimentation Intraperitoneal

1. Le absorption de grassia emulsificate ab le cavitate peritonee esseva demonstrate in le ratto. Tres a 4 ml per 100 g de peso corporee esseva le quantitate maximal de grassia emulsificate que un ratto normal poteva absorber intra 24 horas.

2. Le administration diurne de mesmo solmente 5 ml de grassia emulsificate per 100 g de peso corporee causava sever ascites in le rattos post inter cinque e dece dies. Nulle del rattos recipiente 2,5 ml per 100 g de peso corporee disveloppava ascites.

Oxyuriasis in Laboratory Mouse Colonies

WARREN G. HOAG, D.V.M., M.P.H.

Bar Harbor, Maine

OXYURIASIS in laboratory mice is usually caused by *Aspiculuris tetraptera* and *Syphacia obvelata*.[3, 7] These nematodes are the principal endoparasites which have been found in the inbred mouse colonies of the Roscoe B. Jackson Memorial Laboratory. Morphologically these parasites are distinguishable in adult as well as in egg form.[3, 6] Both are found in the cecum and in the primary portion of the large intestine. Although egg-concentration fecal examination methods, as well as cellophane-tape anal smear techniques, are fairly adequate for diagnostic purposes, our experience has indicated that the necropsy technique used in this report is far more reliable.

The life cycle of these oxyurids is direct, with mature worms developing from ingested eggs within 15 to 30 days. Although rather low incidences in mouse colonies have been reported elsewhere, we have found several colonies with as high as 100 per cent infection.[3, 4, 7] Necropsy examinations have revealed as many as 600 adult worms in the cecum and 1 inch of large intestine adjoining it. With such a heavy intestinal parasitism it seemed unacceptable that no pathologic changes have been attributed to these oxyurids. There have been frequent reports of transported mice either arriving with a high incidence of rectal prolapse or of developing this condition within several days after receipt. Part of these studies were undertaken to determine if any correlation could be established between pinworm infection and rectal prolapse. It was kept in mind that the stress of shipping could play an important precipitating or predisposing role, since the incidence of rectal prolapse in our own colonies (some 60,000 breeding cages) was remarkably low as compared to the high rate of this condition in transported animals. Others have reported upon the successful use of piperazine compounds for ridding mice of oxyurids either experimentally or in small laboratory mouse colonies.[1, 2, 5] Pinworm infection of inbred mice could be assumed to be susceptible to similar treatment but, since the genetic differences among strains of inbred mice are often related to resistance or susceptibility to disease processes, it was considered unsafe to be empirical. A series of experiments was designed to investigate the pathology of oxyuriasis and methods of controlling or eliminating infection.

MATERIALS AND METHODS

Study 1.—A group of 50 C57BL/6J female mice, 6 weeks of age, were earmarked and randomly distributed into two equal groups. Group I mice were given piperazine hexahydrate* in the drinking water at the dosage level of 200 mg./kilogram of body weight. The regimen for this group consisted of daily administration at this dose level for seven days followed by one week on unmedicated water, then seven more days of piperazine-treated water. Group II mice were given unmedicated water. Feed in pellet form was offered ad libitum to mice in both groups. Bedding consisted of pine shavings. All mice were individually weighed at the start of the experiment and weekly thereafter. At the weekly weighing time, mice were changed to washed, sanitized, wooden box-type cages with fresh food in clean hoppers. Daily, each mouse was examined for anal irritation and rectal prolapse. Mice were separately handled by forceps which were alternately rinsed and soaked in a disinfectant solution.** Cages were maintained in a small, disease agent-free isolation room.

At the termination of the experiment (four weeks), all mice were again weighed, then euthanatized and necropsied. Postmortem examination consisted of removal of cecum and of large intestine separately to sterile Petri dishes containing 8 ml. of physiologic saline solution. The tissues were minced with scissors and mixed with the saline solution. This mixture was allowed to stand

Received for publication Feb. 1, 1960.

From the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, where Dr. Hoag is staff scientist and assistant director of production.

This work was supported in part by U.S. Public Health Service grant C-4691.

The author thanks Emmons Brown and Randall Sprague for technical assistance.

* Both piperazine citrate and piperazine hexahydrate supplied by or purchased from Burroughs Wellcome Co.

** Five per cent Wescodyne, produced by West Disinfecting Co.

at room temperature for one half hour before observation. Examination for oxyurids in each Petri dish was then made and the total numbers of oxyurids were recorded (according to intestinal tract location) for each mouse.

Study 2.—Two hundred and twenty-five 6-week-old female hybrid LAF_1 (C57L/J♀ X A/HeJ♂) mice were earmarked and distributed by table of random numbers into three equal groups in wooden box-type cages, each containing 25 mice. Group I mice were given piperazine hexahydrate in the drinking water at a daily dosage of 200 mg. per kilogram of body weight. This was administered for seven days, then unmedicated water was substituted, followed by another week of piperazine citrate in water. These mice were given unmedicated pelleted feed. Group II mice were given piperazine citrate (200 mg./kg. of body weight) which had been premixed and pelleted with the feed. Unmedicated drinking water was available to mice of this group. Group III mice were given unmedicated food and unmedicated water.

All mice were individually weighed weekly and handled as in study 1. Similarly, at the termination of the experiment (six weeks), all mice were weighed, euthanatized, and necropsied; both cecum and large intestine were examined for pinworms.

Study 3.—A colony of approximately 600 cages of inbred mice of 40 strains was selected for this study. These animals were housed in a separate building which had been recently remodeled. Improved methods of sanitation and husbandry were also introduced. The unit was completely isolated, and personnel assigned to it donned sterilized gowns and footgear upon entering. Sanitizing machines in the building made the unit self-sufficient. Clean and dirty traffic and work patterns were also initiated. All of this was intended to maintain a Salmonella-free unit. Upon examination of a sample group of mice from each of the 40 strains (at least 6 animals per strain), it was determined that pinworm infection was prevalent in 100 per cent of the necropsied animals.

The entire colony was treated with piperazine citrate contained in the drinking water at the daily dosage rate of 200 mg./kg. of body weight. As in previous studies, this was administered for seven days, and unmedicated water was given for the next week, followed by another seven days of piperazine citrate water. At six-month and at one-year intervals, a sample number of each strain of mice were necropsied and examined for pinworms as previously described.

Study 4.—A group of 60 LAF_1 female mice, 6 weeks of age, were allotted at random to two groups. All had been tested by the cellophane-tape methods and all were found to be shedding oxyurid eggs. None had evidence of anal irritation or rectal prolapse at the beginning of the experiment. Group I mice were given piperazine citrate in drinking water at the rate of 200 mg./kg. of body weight for seven days, followed by one week of unmedicated water, then seven more days of piperazine treatment. Two weeks later, all mice were euthanatized and at necropsy were examined for presence of pinworms by the aforementioned technique. During the course of the experiment, mice were examined daily for evidence of rectal prolapse and individually weighed weekly.

Each group of 30 mice was housed in a single wooden box-type cage with woven-wire covers holding water bottles and feed hoppers. Pelleted feed was supplied ad libitum. Bedding consisted of pine wood shavings. Cages, covers, and bedding were changed weekly.

Results

Study 1.—All treated animals were free of oxyurids at necropsy (table 1). All untreated mice were infected with both *Syphacia obvelata* and *Aspiculuris tetraptera*. The numbers of each species in cecum and large intestine were not significantly different. The mean number of oxyurids found in mice of this group was 200, with a standard deviation of 75. Rectal prolapse occurred in 2 of the untreated mice but not in the treated mice. No weight differences, either in rate of growth or in terminal weights, could be demonstrated; empirically, however, the treated animals appeared to have better coat condition than the untreated mice.

TABLE 1—Treatment of C57BL/6J Mice with Piperazine Hexahydrate in Drinking Water at a Dosage Level of 200 mg. per Kilogram Body Weight

	No. of infected mice at necropsy	
Oxyurids	Treated mice	Untreated mice
Syphacia obvelata	0/25*	25/25
Aspiculuris tetraptera	0/25	25/25

* Numerator = No. of infected mice; denominator = total No. of mice in group.

Study 2.—Results of study 2 are tabulated (table 2). Since there was no significant weight gain difference between the treated mice and the control mice, this data

TABLE 2—Comparison of the Efficacy of Piperazine Hexahydrate Administered in Drinking Water and in Feed to LAF_1 Mice

		No. infected** at necropsy			
No. of mice	Mode of administration*	Intestine only	Cecum only	Intestine and cecum	Total No. infected
75	food	0	48	15	63
75	water	0	0	0	0
75	controls	4	10	60	74

* Piperazine hexahydrate was given at the rate of 200 mg. per kilogram of body weight. ** Oxyurids present.

has been omitted from the table. At necropsy, the total number of mice found to be infected with oxyurids was not appreciably greater in the group given piperazine hexahydrate in feed pellets than in the group of animals which were untreated. However, it is notable that there was a significant difference in the location of pinworms in the intestinal tracts of treated and control mice. Mice given the drug in the drinking water were completely free of pinworms at necropsy.

Study 3.—All mice which were necropsied six months or one year after treatment with piperazine citrate in drinking water were free of oxyurids. No cases of rectal prolapse were found in any of the mice in the colony thus far during the post-treatment period (15 months).

Study 4.—The results of study 4 are shown (table 3). Five cases of rectal prolapse developed in the infected mice which were not treated. Upon necropsy, all 5 mice were found to be heavily infected with both *S. obvelata* and *A. tetraptera* in both cecum and large intestine. Sagittal tissue sections of the rectums of other mice in the infected group showed varying degrees of irritation, ranging from catarrhal inflammation to sloughing of mucosal cells. The pathogenesis of this condition is under study.

TABLE 3—Incidence of Oxyuriasis and Rectal Prolapse in Treated and Control of LAF_1 mice.

Necropsy finding (3 weeks post-treatment)	No. of mice	No. affected	
		Treated*	Untreated
Oxyuriasis	30	0	29
Rectal prolapse	30	0	5*

* Treated mice were given piperazine citrate in drinking water at the rate of 200 mg. per kilogram of body weight. ** Three of these mice died.

No case of rectal prolapse occurred in the mice treated with piperazine citrate via drinking water. All of these mice were found to be free of oxyurids, and no histopathologic changes were seen in tissue sections of rectums.

DISCUSSION

Apparently both piperazine hexahydrate and piperazine citrate are efficacious in removing oxyurids from mice at the daily dosage level of 200 mg./kg. of body weight. In the one study where piperazine citrate was administered in the feed, the drug seemed to lack efficiency. It can be assumed that the best mode of administration of either drug to mice is through drinking water.

Since these pilot studies were conducted, large-scale use of piperazine citrate has been started in all breeding colonies of the Roscoe B. Jackson Memorial Laboratory. Initially, a single course of the drug was used, based on these experiments; *i.e.*, seven days of piperazine citrate (200 mg./kg. of body weight in drinking water), an intervening seven days of no medication, followed by seven more days of treatment. It was found, however, that unless mice were moved to clean rooms during the course of treatment, reinfection soon took place. Pinworm eggs were found in dust, on equipment, and on the ventilation air-intake ducts of the rooms which had been used previous to treatment. It seems quite obvious that, as in other types of gastrointestinal parasitism, careful environmental sanitation must accompany treatment. The mice in study 3 were given only one course of the drug, yet were free of pinworms 15 months later. This treatment course was accompanied, however, by a complete improvement in sanitation procedures.

Repeated administration (up to 12 courses) of this alternate-week dosage of piperazine citrate via drinking water seems to have no adverse effect on mice of any of our strains, with the exception of some minor strains of mice which consume water at an abnormal rate. It is important that the amount of water consumed daily per mouse be determined so that drug dosage does not reach toxic limits. Since the piperazine compounds produce a colorless solution, it has been found useful to use a small amount of U.S. certified food dye to color the preparation. This has facilitated identification of colonies of mice undergoing treatment.

It is not suggested that rectal prolapse is caused by oxyuriasis, but rather that the rectal irritation accompanying heavy infections predisposes mice to eversion of the rectum. Both shipping stress and the change of diet during this period (potatoes or other vegetables are generally supplied as a source of water) may contribute to the incidence of this condition in pinworm-infected mice.

SUMMARY

Piperazine citrate and piperazine hexahydrate were each effective in ridding inbred and F_1 hybrid mice of oxyurids *(Syphacia obvelata* and *Aspiculuris tetraptera)*. The successful regimen consisted of administering piperazine at a dose of 200 mg./kg. of body weight daily in drinking water for seven consecutive days, succeeded by a period of seven days with no drug, followed by another week of treatment with medicated water. This single course of therapy was most successful when mice were removed to sanitized quarters during or immediately after drug administration.

It is suggested that rectal irritation caused by oxyurid infection may predispose mice to rectal prolapse, particularly when infection is accompanied by the stresses of shipping and drastic change of diet.

References

[1] Brown, H. W., Chan, K. F., and Hussey, K. L.: The Efficacy of Piperazine Compounds Against *Syphacia obvelata*, a Pinworm of Mice. Am. J. Trop. Med. & Hyg., 3, (1954): 504–510.

[2] Fratta, I. D., and Slanetz, C. A.: The Treatment of Oxyurid-Infested Mice with Piperazine Citrate, Stylomycin, Caricide, Phthlofyne, and Gibberellic Acid. Proc. Anim. Care Panel, 8, (1958): 141–146.

[3] Habermann, R. T., Williams, F. P., Jr., and Thorp, W. T. S.: Identification of Some Internal Parasites of Laboratory Animals. U.S. Public Health Service Pub. 343, 1954.

[4] Habermann, R. T., and Williams, F.: The Effects of Antibiotics, Phenothiazine, Sodium Fluoride, and the Combined Action of These Drugs in the Removal of Oxyurids in Mice. Proc. Helminthol. Soc. Washington, 23, (1956): 36–39.

[5] Habermann, R. T., and Williams, F.: The Efficacy of Some Piperazine Compounds and Stylomycin in Drinking Water for the Removal of Oxyurids from Mice and Rats and a Method of Critical Testing of Anthelmintics. Proc. Anim. Care Panel, 7, (1957): 89–97.

[6] Philpot, F.: Notes on the Eggs and Early Development of Some Species of Oxyuridae. J. Helminthol., 2, (1924): 239–252.

[7] Thompson, P. E., and Reinertson, J. W.: Chemotherapeutic Studies of Natural Pinworm Infections of Mice. Exptl. Parasitol., 1, (1952): 384–391.

SUMMARIO IN INTERLINGUA

Oxyuriasis in Colonias de Muses Laboratorial

Citrato de piperazina si ben como hexahydrato de piperazina esseva efficace in eradicar oxyurides *(Syphacia obvelata* e *Aspicularis tetraptera)* in muses ab parentage consanguinee e in muses hybrida F_1. Le efficace regime consisteva in administrar piperazina in un dosage de 200 mg per kg de peso corporee per die in aqua potabile durante periodos de septe dies consecutive, sequite per un periodo de septe dies sin le droga e postea un secunde septimana con le medication del aqua. Iste curso de therapia succedeva le melio quando le muses esseva transferite in quartieros sanitarisate durante o immediatemente post le administration del droga.

Es proponite le these que le irritation rectal que es causate per un infection oxyuridic predispone le muses a prolapso rectal, specialmente si le infection es accompaniate per le stresses de transporto e alterationes drastic in le dieta.

Scientific Notes

Study of Chicken Sarcoma (Strain 13) Placed in the Bursa of Fabricius

E. L. STUBBS, V.M.D., and F. G. SPERLING, V.M.D.

Philadelphia, Pennsylvania

STUDIES WITH a transmissible chicken sarcoma (strain 13) prompted experiments to determine whether this tumor would grow in the bursa of Fabricius, and because this is a virus-induced tumor, whether any type of resistance would be stimulated by exposure in the bursa. Chicken sarcoma (strain 13) has been reactivated from tumor material desiccated from the frozen state and kept for 20 years.[5] The bursa of Fabricius has been used to produce resistance to infectious laryngotracheitis, a viral disease,[1] and has been shown to build up antibodies against Salmonella infections.[6]

The bursa of Fabricius, found only in birds, is composed of lymphoid tissue connected by a groove to the dorsal cloaca. The bursa has been shown to be associated with growth and maturity.[4] It has been shown also to be associated with antibody production, because bursectomized chickens were more susceptible to Salmonella typhimurium infection and did not build up antibodies in the blood as did non-bursectomized chickens.[6]

Glick et al.[3] reported that the bursa reaches its maximum size in four to five weeks in White Leghorns and as late as nine weeks in Rhode Island Reds. These same workers [3] consider this early life as the time that chickens develop the ability to produce antibodies against foreign proteins and that resistance, in general, may be associated with the growth rate and size of the bursa during the time when the bird develops the ability to produce antibodies. Other workers [2] have shown that the effect of antibody production was greatest during the first weeks after hatching and declined with increasing age; the antibody titer following a single injection of antigen was higher after seven than after four, 11, or 18 days.

Chicken sarcoma (strain 13), carried in young chickens by routine transplantation [5] was available for experiments. This tumor is easily transmitted from chicken to chicken when minced tumor tissue, suspended in physiologic saline solution, or a filtrate of this material is injected intramuscularly into chickens. A tumor appears at the point of injection, grows rapidly, becomes highly malignant, and kills young chickens. When this tumor spreads, it is found in the liver, spleen, and, occasionally, the bone marrow. Since this is a virus-induced tumor and the bursa of Fabricius seems to be important in producing general resistance, especially in the formation of antibodies, placing tumor material in the cloaca and bursa might result in tumor growth or the production of resistance. It would be useful to know if this tumor would grow in the bursa; furthermore, any resistance to chicken sarcoma could be determined by intramuscular challenge.

PROCEDURES and RESULTS

In the first experiment, 15 chicks were used. Tumor pieces were removed from a rapidly growing tumor, placed in a Petri dish on ice, and minced. The minced tumor tissue, suspended in physiologic saline solution, was introduced into the cloaca and pushed with an applicator stick along the dorsal groove into the bursa of Fabricius. The chicks were examined each week. At the fourth week after exposure no tumors had developed, and the immunity of each was challenged by injecting fresh tumor material into the breast muscle. All the chicks developed tumors in the breast muscles.

In the second experiment, 9 chicks were exposed in a similar manner, using chicken sarcoma material from a tumor established one month previously in a donor. The donor chicken was affected with a soft, rapidly growing tumor and was killed for transfer purposes. Opaque, gray, soft, tumor tissue was minced and introduced into the cloaca and pushed by an applicator stick into the bursa of Fabricius with attempts to bruise the bursa. No tumors were detected and, after one month, the immunity of each chick was challenged by intramuscular injection of fresh tumor suspension. All chicks developed tumors.

The third experiment was designed to try two exposures, one at hatching time and the other three weeks later. This was done to allow a longer period for the production of any resistance while the bursa was developing, as suggested by other workers.[6] A group of 50 newly-hatched chicks was selected. Of 20 caged together, 10 were exposed by application in the cloaca and bursa, and the

Received for publication June 22, 1960.

From the School of Veterinary Medicine, University of Pennsylvania, Philadelphia.

This work was aided by the Goldhaft Poultry Research Fund and by grant C-4610 from the U.S. Public Health Service.

other 10 served as controls. Another 10 were exposed by intramuscular injection of the tumor suspension to determine its activity and the susceptibility of the chicks. These 10 developed tumors, but the 10 exposed via the bursa and the 10 untreated controls did not. Three weeks after the initial exposure, when none of the bursa-exposed chicks had any tumor growth, each was exposed again by introducing tumor suspension into the bursa of Fabricius. At the same time, another 10 of the original chicks, now 3 weeks old, were exposed intramuscularly to determine the activity of the exposure material. Two weeks after the second exposure, considered the optimum time for antibody production to occur, the immunity of the 10 chicks which were exposed twice was challenged by intramuscular injection. At the same time, the 10 remaining chicks of the original 50 were given injections of the tumor suspension used for challenge. No differences could be found in the size or rapidity of tumor growth resulting from intramuscular injection in the twice-exposed chicks as compared with any of the controls. The three trials indicated that this tumor failed to grow in the bursa of Fabricius and no evidence of any resistance could be found even after two experimental exposures.

SUMMARY and CONCLUSIONS

Three experiments were conducted in an attempt to grow a transmissible chicken sarcoma (strain 13) in the bursa of Fabricius. No tumor growth resulted in the chicks tested.

Since chicken sarcoma (strain 13) is a virus-induced tumor, expriments were designed to determine whether any resistance could be produced by bursal exposure. A single intrabursal exposure was used for chicks in two experiments; those in the third experiment were subjected to a second exposure three weeks after the first. Following exposure, the chicks' immunity was challenged by injection of fresh tumor suspension intramuscularly. In the first two experiments, the challenge was made one month after exposure. In the third experiment, challenge was made two weeks after the second exposure, when the antibody p. duction should have been at its peak. The fresh tumor suspension was checked for activity by intramuscular injection in groups of control chicks.

Tumors grew in the principals and controls alike. No differences could be detected in size of the tumors, rapidity of growth, or effects on the birds. It was concluded that the bursa of Fabricius is unsuitable for growth of this tumor and that no resistance had been produced by the intrabursal application of fresh tumor material.

References

[1] Beaudette, F. R., and Hudson, C. B.: Experiments on Immunization Against Laryngotracheitis in Fowls. J.A.V.M.A., 82, (1933): 460–476.

[2] Chang, T. S., Rheims, M. S., and Winter, A. R.: The Significance of the Bursa of Fabricius in Antibody Production in Chickens. Poult. Sci., 36, (1957): 735–738.

[3] Glick, Bruce, Chang, Timothy S., and Jaap, R. George: The Bursa of Fabricius and Antibody Production. Poult. Sci., 35, (1956): 224–225.

[4] Riddle, O.: Growth of the Gonads and Bursa of Fabricius in Doves and Pigeons with Data for Body Growth and Age at Maturity. Am. J. Physiol., 86, (1928): 243–265.

[5] Stubbs, E. L. and Sperling, F. G.: Desiccated Chicken Sarcoma Viable for Twenty Years. J.A.V.M.A., 136, (1960): 376–377.

Summario in Interlingua

Un Studio de Sarcoma de Gallina (Racia 13), Placiate in le Bursa de Fabricius

Tres experimentos esseva executate in le effortio de mantener un transmissible sarcoma de gallina (racia 13) in le bursa de Fabricius. Nulle crescentia tumoral resultava in le gallinettas testate.

Viste que sarcoma de gallina (racia 13) es un tumor inducite per virus, experimentos esseva elaborate pro determinar si il esseva possibile producer resistentia per exposition bursal. Un sol exposition intrabursal esseva usate in gallinettas in duo experimentos. In un tertie experimento, le gallinettas esseva subjicite a un secunde exposition tres septimanas pose le prime. Post le exposition, le immunitate del gallinettas esseva provocate per injectiones intramuscular de un suspension de tumor fresc. In le prime duo experimentos, le provocation esseva effectuate un mense post le exposition. In le tertie experimento, le provocation esseva effectuate duo septimanas post le secunde exposition, a un tempore quando le production de anticorpore deberea haber attingite su culmine. Le suspension de tumor fresc esseva probate pro le potentia de su activitate per injection intramuscular in gruppos de gallinettas de controlo.

Le tumores cresceva in le aves pretractate precisemente como in le aves de controlo. Nulle differentia esseva detegibile in le dimensiones del tumores, le rapiditate del crescentia del tumores, o le effectos del tumores super le aves. Il esseva concludite que le bursa de Fabricius non es usabile pro le crescentia de iste tumor e que nulle resistentia habeva essite producite per le application intrabursal de fresc material tumoral.

Cardiac Output and Related Values in Anesthetized Deer

A. L. GOOD, V.M.D., Ph.D.; C. M. STOWE, V.M.D., Ph.D.; LYTLE BLANKENSHIP, Ph.D.

St. Paul, Minnesota

THERE apparently is no published information regarding cardiovascular function in the white-tailed deer (Odocoileus virginianus var. borealis). This work was undertaken in an attempt to provide limited data of a comparative nature in this species. Some of the animals used in the study were orphans which had been obtained from the wild by Department of Conservation personnel and brought to a game farm near Forest Lake, Minn. These animals were raised in outdoor pens and were handled frequently. Even though they were friendly, general anesthesia was necessary to permit the necessary experimental procedures. The older deer used in the study were nuisance animals that had been removed from a semiwild environment and brought directly to the veterinary college.

METHODS

Five healthy fawns born in the spring of 1958, 1 doe 12 months old, 1 buck 18 months old, and 1 doe 24 months old were used in the study. All except deer 1 were anesthetized with chloral hydrate to plane I or II of surgical anesthesia. Deer 1 was given thiopental sodium. The anesthetic was administered intravenously.

The right ventricle was catheterized with polyethylene tubing via the external jugular vein. Catheter location was judged by the pressure observed with a mercury manometer. The aorta was punctured with a 6-inch, 14-gauge needle through the left dorsolateral region of the last intercostal space. Following rapid injection of 0.25 to 0.35 mg./kg. of T-1824 (Evans blue dye) through the cardiac catheter, aortic blood was collected via a polyethylene catheter suspended above a series of test tubes containing sodium oxalate crystals. The test tubes, of known diameter, were tightly strapped to the circumference of a kymograph drum which was rotating at a known velocity (fig. 1). In this manner, a separate blood sample was collected every 1.43 seconds. After centrifuging the individual samples and withdrawing the plasma, the dye concentration in each sample was determined in a Beckman Model DU spectrophotometer at 620 mμ. A standard curve was prepared by placing known amounts of dye in plasma previously collected from each animal.

A curve showing the concentration of dye in the plasma samples was prepared on semilogarithmic paper by plotting time in seconds on the abscissa and the dye concentration on the logarithmic scale of the ordinate. The straight line, representing the logarithmic decrease of dye concentration with time, was extrapolated to the abscissa, thus permitting an estimate of the time duration of the dye curve to be made. The average concentration of dye over this time period was determined, and the cardiac output calculated according to the formula:

$$F = \frac{60 \times A}{T \times C \times (1-H)}$$

Where F is cardiac output in liters per minute, A is the amount of dye injected in milligrams, T is the time in seconds from the first appearance of dye to the interception of the extrapolated downslope of the curve to the abscissa, C is the mean dye concentration in milligrams per liter, and H is the hematocrit value. A typical dye curve, obtained from deer 2, is shown (fig. 2). The method employed is similar to that reported by Hamilton et al.[3]

Aortic and right ventricular mean pressures were measured with a mercury manometer. Packed cell volume determinations were made by placing the blood in a Wintrobe tube and centrifuging for 30 minutes at 2,500 r.p.m. Blood volume was estimated by determining the plasma concentration of T—1824 at 10, 20, and 30 minutes after dye injection, and extrapolating the resultant straight line (semilogarithmic plot) to zero time. A correction was made for the volume of plasma trapped between erythrocytes.[4]

RESULTS

The results of the cardiac output estimates, blood pressure, blood volume, and calculated peripheral resistance are shown (table 1). These data are presented with a view to supplying some approximate values of a preliminary nature in deer, rather than presenting data which represent a true mean for the species. For example, deer 1 was taken directly from the wild under considerable harassment. This may explain the relatively high cardiac output and blood pressure observed. However, there is no simple explanation for the relatively low output and aortic pressure in deer 3. In view of the rather diverse background of the animals and the limited number employed, statistical treatment of the data would be of limited value.

Received for publication Jan. 11, 1960.

From the Division of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, University of Minnesota, and the Section of Research, Minnesota Division of Fish and Game, St. Paul.

Presented in part before the Section on Research, 96th Annual Meeting, American Veterinary Medical Association, Kansas City, Mo., Aug. 24–27, 1959.

The authors thank B. A. Fashingbauer, John Idstrom, and others from the Minnesota Department of Conservation, and Frederick Borgwardt, Glenn Wolf, and Chris Nissen, students at the College of Veterinary Medicine, University of Minnesota, who assisted in this project.

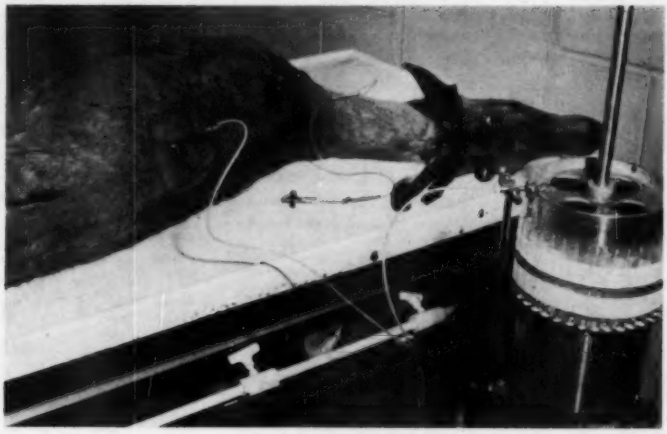


Fig. 1—Arrangement of equipment for estimating the cardiac output of deer.

Deer 1 was anesthetized with thiopental sodium, which is classed as an ultrashort-acting barbiturate in dogs and in man (15 to 30 min.). Deer 1 remained in surgical anesthesia for several hours following induction; 36 hours elapsed before all signs of ataxia disappeared and the animal had completely recovered. Perhaps the response is similar to that of calves, in which thiopental may produce prolonged general anesthesia.

All of the remaining deer were anesthetized with chloral hydrate; response to the drug was similar to that of cattle. The drug was administered to effect; the amount required to attain plane I of surgical anesthesia was approximately 6 Gm./100 lb. of body weight (130 mg./kg.). Supplementary doses of chloral hydrate were required in some animals to maintain the proper depth of anesthesia during the experiments. When the various procedures were completed, the animals were permitted to recover. This normally took one to one and one-half hours and was characterized by little struggling or ataxia. One deer, not included in this report, died during chloral hydrate anesthesia.

DISCUSSION

The average cardiac output of 114 ml./kg./min. and the range of 81 to 171 ml./kg./min. observed in these deer are similar to values obtained in both anesthetized and unanesthetized calves and anesthetized ewes.[5] Table 2 shows the range and mean values obtained by the same method in the other two ruminant species studied. Initially, it was thought that deer would have a higher cardiac output than domestic ruminants of comparable size and weight. Although such an assumption may be correct, these data, obtained under so-called "basal" conditions, suggest that deer, calves, and

TABLE 2—Cardiac Output in Three Ruminant Species *

Species	No.	Cardiac output (mg./kg./min.) Mean	Range
Anesthetized ewes (pentobarbital Na)	6	126	93–163
Anesthetized calves (pentobarbital Na)	9	119	82–169
Unanesthetized calves	15	121	97–153
Anesthetized deer	8	114	81–171

* Data on calves and sheep obtained from Stowe and Good.[5]

TABLE 1—Hemodynamic Observations in Anesthetized Deer

Deer No.	Age (mo.)	Sex	Wt. (kg.)	Cardiac output (ml./kg./min.)	Mean pressure (mm. Hg) Rt. vent	Aorta	Periph. resist. (abs. units)	Plasma vol. (liters)	Packed cell volume	Blood vol. (ml./kg.)
1*	12	F	47.7	171	20	155	1520	.....	34.0	....
2	5	M	21.4	136	15	120	3320	.....	34.0	....
3	6	M	23.6	81	16	63	2620	1.04	27.8	60
4	18	M	65.9	113	12	115	1240	3.36	25.1	67
5	9	M	42.8	136	10	96	1320	1.49	50.5	73
6	8	M	28.6	95	10	66	1950	1.03	43.6	61
7	24	F	60.0	96	....	140	1953	.....	35.0	....
8	10	....	40.4	87	....	75	1800	.....	37.8	....
	Mean		41.3	114	14	104	1965	1.73	35.9	65

* Anesthetized with thiopental sodium, 40 mg./kg. intravenously.

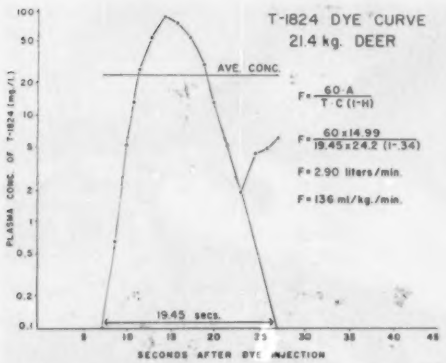

T-1824 DYE CURVE
21.4 kg. DEER
AVE. CONC.
F = 60 A / T C (I-H)
F = 60 x 14.99 / 19.45 x 24.2 (1-.34)
F = 2.90 liters/min.
F = 136 ml/kg./min.
19.45 secs.
PLASMA CONC. OF T-1824 (mg./l.)
SECONDS AFTER DYE INJECTION

Fig. 2—A typical curve obtained by plotting the plasma dye concentration against time in a 21.4-kg. deer.

sheep have comparable cardiac outputs.[5] Further studies to clarify this point are underway.

Blood volume estimates from 4 of the 6 deer averaged 65 ml./kg. of body weight. This value is approximately 14 per cent greater than that reported for cows by Reynolds[4] and about 16 per cent lower than that obtained by Mizuno for calves.[3]

The total peripheral resistance, calculated from the mean blood pressure and cardiac output,[1] was found to vary inversely with the size of the animal. It amounts to about 600 to 2,000 absolute units in man, 2,000 to 9,000 in the dog, and 11,500 to 12,000 in the rabbit.

SUMMARY

The dye dilution technique for estimating cardiac output was applied to deer. The average cardiac output for 8 deer weighing 21 to 66 kg. was 4.7 liters/min. (114 ml./kg./min.). The mean aortic blood pressure was 104 mm. Hg, while the mean right ventricular pressure was 14 mm. Hg. The calculated peripheral resistance was of the same order of magnitude as that for man.[1]

REFERENCES

[1] Best, C. H., and Taylor, N. B.: The Physiological Basis of Medical Practice. Williams & Wilkins Co., Baltimore (1955): 148.

[2] Hamilton, W. F., Riley, R. L., Attyah, A. M., Cournand, A., Fowell, D. M., Himmelstein, A., Noble, R. P., Remington, J. W., Richards, D. W., Wheeler, N. C., and Witham, A. C.: Comparison of the Fick and Dye Injection Methods of Measuring the Cardiac Output in Man. Am. J. Physiol., 153, (1948): 309–321.

[3] Mizuno, N., University of Minnesota, St. Paul: Personal communication, 1959.

[4] Reynolds, M.: Plasma and Blood Volume in the Cow Using the T-1824 Hematocrit Method. Am. J. Physiol., 173, (1953): 421–427.

[5] Stowe, C. M., and Good, A. L.: The Estimation of Cardiac Output in Calves and Sheep by the Dye and Fick Oxygen Techniques. Am. J. Physiol., 198, (1960): 987–990.

SUMMARIO IN INTERLINGUA

Le Rendimento Cardiac e Relationate Parametros in le Anesthesiate Cervo

Le technica a dilution de colorante pro le estimation del rendimento cardiac esseva applicate a cervos. Le rendimento cardiac medie in 8 cervos de inter 21 e 66 kg de peso corporee amontava a 4,7 litros per minuta, i.e. 114 ml per kg per minuta. Le tension sanguinee medie esseva 104 mm de Hg, durante que le tension dextero-ventricular medie esseva 14 mm de Hg. Le calculate resistentia peripheric esseva del mesme ordine de magnitude como in le homine.

Exsanguination of Chickens by Catheterization

ARTHUR A. HIRATA, Ph.D.

Pasadena, California

A METHOD of exsanguinating chickens was developed for two purposes: (1) to obtain blood samples uncontaminated by tissue juices and (2) to obtain the maximum amount of blood from a single chicken.

A chicken was restrained in a supine position with one wing spread out. An 18-gauge, thin-walled hypodermic needle was inserted into a branch of the brachial vein which crosses the proximal end of the ulna. A polyethylene tube (35 cm. long, with an inside diameter of 0.58 mm. and an outside diameter of 0.965 mm.) was inserted through the hypodermic needle into the brachial vein and advanced to the vicinity of the heart. The distance between the point of insertion and the heart measured approximately 13.0 cm. in a 2-kg. White Leghorn.

Blood was collected in a test tube fitted with a rubber stopper, through which a glass tube and an 18-gauge, thin-walled hypodermic needle were inserted. The polyethylene tube was inserted into the

test tube through the hypodermic needle in the stopper. Then the glass tube was connected to an aspirator (approximately 715 mm. Hg). Usually, blood started to flow into the test tube immediately. If the flow did not begin immediately, the polyethylene tube was pushed in or out about a centimeter to secure the proper position in the vicinity of the heart. The initial flow rate was about 5 ml. per minute. After 80 to 90 ml. was obtained, the flow rate decreased to about 2 ml. per minute. The total amount of blood obtained from 2-kg. White Leghorns was 110 to 125 ml., with an average of about 120 ml. In contrast, yields of only 50 to 75 ml. have been obtained by the heart-puncture method.

Catheterization of the brachial veins of the right and left wings gave similar results.

About half of the blood samples obtained by catheterization clotted within 60 minutes and, when centrifuged, yielded 50 to 60 per cent serum. However, blood samples with a clotting time longer than 60 minutes gave low serum yields (2–20%) due to the formation of plasma clots. The adlition of a drop of chicken thromboplastin reagent * per 10 ml. of blood shortened the clotting time and prevented the plasma clot formation.

For mass sampling, two sets of bleeding apparatus were used simultaneously; 10-ml. blood samples were obtained at a rate of five minutes per bird.

Received for publication July 1, 1960.

From the Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, Calif., published as contribution No. 2624. Dr. Hirata, National Science Foundation postdoctoral fellow, is now with the Laboratory of Chemical Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, Md.

* Hawk, P. B., Oser, B. L., and Summerson, W. H.: Practical Physiological Chemistry. 13th ed. McGraw-Hill Book Co., New York, N.Y., (1954): 490.

SUMMARIO IN INTERLINGUA

Exsanguination de Gallinas per Catheterismo

Un methodo pro le exsanguination de gallinas per catheterismo es describite. Le avantages del methodo es (1) que illo permitte le obtention de specimens de sanguine sin contamination per succos tissular e (2) que illo resulta in un rendimento plus grande de sanguine ab le gallina individual.

Le catheter es avantiate a in le vicinitate del corde. In le caso de leghorns blanc de 2 kg de peso, le redimento medie esseva circa 120 ml de sanguine per gallina. Le methodo de puncturation cardiac produce solmente inter 50 e 75 ml de sanguine per gallina.

Research Tools

Further information on these products may be obtained by writing to: Research Tools, American Veterinary Medical Association, 600 S. Michigan Ave., Chicago 5, Ill.

Radioactivity Measuring Device

A new device for measuring radioactivity (Magnascaler, manufactured by the Picker X-Ray Corp.) can be used with scintillation detectors, Geiger tubes, and proportional counters with pre-amplifiers. The unit collects more than 600,000 counts per minutes with an accuracy of 99 per cent.

A built-in odometer-type timer measures up to 999.99 in steps of 0.01/minute and is accurate within 0.02 of 1 per cent. The instrument can be pre-set in 11 time positions and 11 count positions. Counts can be read directly from six electronic decades. The unit can be combined with automatic sample changers and printers to read out time and count data.

Home Air Purifier

A new type of home air purifier, based on the principle of electrostatic precipitation, has been developed by Granco Products, Inc. The purifier clears the air of foreign materials such as dust, pollen, soot, fumes, and odors, while it revitalizes the purified air by adding a flow of negative ions. Previously, this method was limited to large installations such as hospitals, laboratories, and industrial plants.

This new purifier is built around an electronic energizer and control unit. It has four electron tubes to charge the incoming air to a high potential and then pulls foreign matter out by applying an equal and opposite charge to a series of plates with a filter surface of more than 24 square feet.

A three-speed fan circulates all air in a 20-by 20-ft. room through the filter every two minutes. Self-contained, the unit is portable and usable in any room or office with a 110-volt outlet. It uses no more power than a 40-watt electric light bulb.

CO_2 Tissue Culture Incubator

A stainless steel incubator for growing single cells in CO_2 atmosphere, with or without humidity, has been developed by Labline, Inc. The desired humidity is provided by bubbling CO_2 through a water bottle, thereby adding moisture to the chamber and preventing the drying of cultures.

The incubator has a built-in blower and "Thermo-wall" heating system for maintaining uniform chamber temperatures. A Powerstat control regulates the speed of the blower, thus varying the rate of air flow through the chamber. A double-latch door with glass window and cover assures accurate temperatures at all times.

The exterior cabinet is made of stainless steel with Monel metal interior. It has double-wall construction with an inch of glass wool insulation throughout.

Cardiac Monitor

A cardiac monitor instrument translator, designed to monitor electrocardiographic signals during surgery, postoperative recovery periods, and in the study of biological functions in test animals, is now being produced by Crescent Engineering and Research Co.

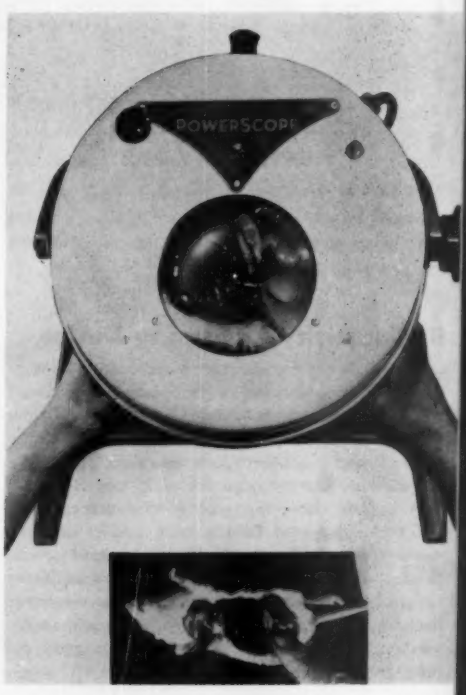
Signals may be read from the instrument meter, recorded on a suitable recorder, or observed on an oscilloscope.

The 4-lb. unit is about 7 inches long, 4 inches high, and 3 inches wide. Housing and panel are aluminum with an enamel finish.

"Powerscope" for Macro-Viewing

The "Powerscope," a self-illuminated magnifying optical system for macro-viewing up to 7 times life size without distortion, shadows, or color aberrations, has been developed by the Ednalite Optical Company, Inc.

This optical system has a 5-inch diameter viewing aperture and permits fatigue-free viewing with both eyes. The special lens amplifier swings in to double normal power for extra-critical localized viewing. Adjustable viewing positions utilize full depth-of-field capabilities and allow in-focus work with full freedom of hands and tools beneath viewing system.



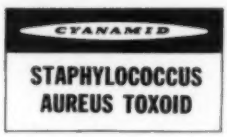
In the one year since its introduction, many veterinarians with dairy cattle practice have made Staphylococcus Aureus Toxoid (Slanetz Strain) a standard procedure in their mastitis control work.

Cyanamid advertises this veterinary service to dairymen . . . urges them to check with their local veterinarian for a practical control program.

And now, for the first time in months, adequate supplies of Staphylococcus Toxoid are on hand to fill all orders.

Staphylococcus Aureus Toxoid (Slanetz Strain) is available in 50 cc. or 250 cc. vials from your Veterinary distributor. The dose is 5 cc., I.M., repeated in one month and annually thereafter.

Veterinary Professional Service Department
American Cyanamid Company
New York 20, New York



ANNOUNCING
A SIGNIFICANT ADVANCE
IN THE PREVENTION OF
CANINE DISTEMPER

TISSUVAX D-H*

the vaccine that produces
higher levels of protection
against DISTEMPER
than ever before reported

A Pitman-Moore development in tissue culture research.

*Trademark for CANINE DISTEMPER VACCINE, Modified Live Virus, Chick Tissue Culture Origin, and INFECTIOUS CANINE HEPATITIS VACCINE, Modified Live Virus, Porcine Tissue Culture Origin, Combined, Vacuum Dried

PITMAN-MOORE COMPANY
DIVISION OF ALLIED LABORATORIES, INC. • INDIANAPOLIS 6, INDIANA